EPIDEMIOLOGY OF *FASCIOLA HEPATICA* IN FLORIDA WITH EMPHASIS ON THE POPULATION DYNAMICS AND INFECTION PREVALENCE OF THE PRIMARY SNAIL INTERMEDIATE HOST, *FOSSARIA CUBENSIS*

By

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Fascioliasis is one of the most important diseases of cattle in Florida. Southern Florida, where more than 66% of beef cows are pastured, is subtropical, and conjectural evidence suggests that seasonal transmission of *Fasciola hepatica* may differ from that in temperate northern Florida. Current recommendations for control of fascioliasis in cattle are based on data only from northern Florida. Therefore, it is extremely important to the cattle industry of southern Florida to determine the seasonal transmission dynamics of *F. hepatica*. In this project, the epidemiology of *F. hepatica* in northern, central and southern Florida was determined over 2 years by studying the bionomics of *Fossaria cubensis* populations on 7 cattle ranches. Additionally, snail infection prevalence was determined during year 2 of this

study. Because existing techniques lacked the sensitivity and specificity needed to obtain accurate infection prevalence data, a DNA probe was developed. This DNA probe (pFh5) contains 2 124 bp repeat sequences belonging to a large family of 124 bp repeats constituting approximately 15% of the *F. hepatica* genome. pFh5 has excellent sensitivity and specificity; a single miracidia is detected and it does not cross hybridize with DNA of *Fascioloides magna*, *Paramphistomum liorchis* or *Heterobilharzia americana*, trematodes that share the same intermediate host and enzootic range as *F. hepatica*. Using this DNA probe, over 5,000 snails from 6 ranches were assayed. Infection prevalence for individual ranches varied from 0.1% to 3.1%. In a separate experiment, it was demonstrated that eggs of *F. hepatica* can survive and develop on pasture during the summer with greater than 80% survival after 28 days.

Results of this study demonstrated that seasonal transmission of liver flukes is virtually the same for all of Florida. Transmission occurs predominantly in winter and spring. Wet summers followed by cool, wet weather in early autumn could result in significant levels of transmission occurring by December. Summer transmission will rarely occur in Florida. Although snail habitats remain wet throughout summers of most years, ecological conditions of *Fossaria* habitats are not conducive to snail activity. Therefore, current control recommendations remain unchanged except that

annual treatment should be given in late summer, 1 to 2 months sooner than recommended previously.

CHAPTER 1
INTRODUCTION

History and Distribution of *Fasciola hepatica*

Fasciola hepatica, the common liver fluke, belongs to the phylum Platyhelminthes, class Trematoda, order Digenea, family Fasciolidae. Domestic ruminants are the definitive hosts, however numerous other mammals, including man, may become infected (Malek, 1980). The earliest known reference to the liver fluke (and liver rot) was made in 1379 by Jean de Brie in France, in his treatise on the proper management of sheep. Sir Anthony Fitzherbert published the first recognizable description of the liver fluke in 1523, and Francesco Redi in 1668 published the first illustration. A major breakthrough in the understanding of trematode biology came in 1842, when J. Steenstrup published his work on the theory of "alternation of generations". This work drew wide attention and paved the way for the independent discovery of the complete extra-vertebrate life cycle of *F. hepatica* by A.P. Thomas in England and R. Leuckart in Germany in 1882. The discovery of the migratory path taken by larval flukes in their vertebrate hosts was made by D.F. Sinitsin in 1914 in Russia, thus completing the knowledge of the liver fluke life cycle (Reinhard, 1957).

Fascioliasis, caused by infection with the liver fluke, *Fasciola hepatica*, remains one of the most important diseases of grazing ruminants throughout much of the world (Malek, 1980). The transmission of *F. hepatica* is dependant upon the presence of its lymnaeid snail intermediate hosts, therefore the distribution of the parasite is limited to those geographic areas where the appropriate snail species are present. In the United States, *F. hepatica* is enzootic primarily in the Gulf coast and western states, where high annual rainfall, large areas of poorly drained pasture and certain soil types provide suitable lymnaeid snail habitats (Malone, Loyacano, Armstrong, & Archbald, 1982a). Additionally, the distribution and prevalence of liver flukes may increase in areas where irrigated pastures are used. This is particularly important in the western United States (Malczewski, Wescott, Spratling, & Gorham, 1975). In Florida, *F. hepatica* is almost exclusively restricted to the peninsula south and east of the Suwannee River (Shearer, Courtney, & Richey, 1986) where 92% of Florida's 1.1 million beef brood cows are pastured (Florida Department of Agriculture and Consumer Services, 1994).

Biology and Life Cycle of *Fasciola hepatica*

Adult *F. hepatica* reside in the intrahepatic biliary ducts of host animals and eggs are carried with the bile into the bowel lumen and then passed in the feces. Each of these eggs

contains a fertilized ovum which develops into a ciliated larva called a miracidium. The rate of miracidial development is dependent on temperature and oxygen availability and can take from 10 days to several months. The critical temperature below which egg development ceases is 9.5°C and the thermal constant for the optimal temperature range for egg development is between 200 and 220 day-degrees, the values obtained when eggs are incubated at 23°C and 18°C respectively. The maximum temperature that eggs can be exposed to and still survive is not known although temperatures above 30°C increasingly inhibit development. Additional requirements for successful development and hatching of eggs include separation from the feces, and a film of moisture on the egg surface during the entire developmental period from deposition onto pasture until hatching of the free-swimming, ciliated miracidia (Rowcliffe & Ollerenshaw, 1960). Once the miracidium is fully developed, exposure to sunlight stimulates it to hatch, although the precise mechanism of hatching has not been proven. Roberts (1950) demonstrated that violet and blue wavelengths of the light spectrum are an essential part of the light stimulus. Rowan (1956) suggested that the operculum is cemented to the shell and that the miracidium releases an enzyme at the time of hatching that digests this cementing substance. Wilson (1968) refuted this explanation and suggested that the miracidium causes an alteration of the permeability of the membrane surrounding the viscous cushion. This change in

permeability causes an expansion of the cushion which compresses the miracidium, leading to an increase in the internal pressure within the egg until the operculum finally ruptures. Both studies demonstrated that the escape of the miracidium from the open egg is due primarily to the hypertonicity of the egg contents which forcefully expels the miracidium, and only secondarily to muscular activity.

After hatching, the miracidium has only a few hours in which to find and penetrate a suitable snail host. During the process of penetration, the miracidium loses its ciliated covering and becomes a sporocyst. The sporocyst, which has a rudimentary digestive tract and is filled with germinal cells, is usually found in the wall of the mantle cavity, the wall of the pulmonary chamber, or in the tissue around the esophagus (Kendall, 1965). The germinal cells within the sporocyst develop into rediae, the next larval stage. Within 2 to 3 weeks the mature rediae leave the sporocyst by rupturing the wall and migrate to the digestive gland (hepato-pancreas). Rediae may then produce either a daughter generation of rediae or cercariae depending upon various environmental stimuli. Stresses such as snail host starvation and high or low temperature tend to stimulate daughter redial production (Wilson & Draskau, 1976). The tadpole-like cercariae leave the rediae through a birth pore and enter into the perivisceral space before emerging from the snail. Cercarial emergence is a mostly passive process caused by an increase in

pressure in the perivisceral spaces. This rise in pressure results from contractions of the walls of the mantle cavity which occur with increased snail activity. Immersion of the snail in fresh water appears to be the principal factor governing this process (Kendall and McCullough, 1951). This has important epidemiologic implications since cercariae will not be shed if the environment is dry when dispersion and survival of the cercariae would be unlikely. Cercariae have been observed to emerge in large numbers following rain that was preceded by a dry period (Boray, Happich, & Andrews, 1969). Infection of a snail with a single miracidium can result in the production of hundreds of cercariae and this reproductive process greatly contributes to the success of this parasite. The rate of development of these intramolluscan stages is dependent upon temperature and the nutritional state of the snail (Kendall, 1965). Under optimal conditions, parasite maturation within the snail takes approximately 5 to 7 weeks.

The free-swimming cercariae loses its tail after contacting vegetation, secretes a protective cyst covering, and completes its development during the first 2 or 3 days after encystment (Malek, 1980). This stage, the metacercariae, contains a fully developed immature fluke and is the infective stage of the parasite. Ruminant hosts become infected primarily by ingesting the metacercarial cysts on forage, but they also can become infected by ingesting cysts suspended on soil and

detritus while drinking contaminated water. The length of time that metacercariae survive on pasture is dependant on environmental factors. Moisture is the principal factor controlling the length of life of metacercariae, with a minimum of 70% relative humidity considered necessary for prolonged survival. Metacercariae were killed within two days of exposure to temperatures of 98.6 to 105°F when exposed in air to direct sunlight, however when placed in water kept at room temperatures ranging from 71.6 to 80.6°F they survived for 4 months. In Great Britain, metacercariae shed onto pasture in the autumn remained infective for periods of between 270 to 340 days and metacercariae shed onto pasture during the summer remained viable for up to 180 days (Kendall, 1965). Under the hot and dry pasture conditions of coastal Texas during the summer, metacercariae were rapidly killed (Olsen, 1947), however under conditions of high humidity such as exist in Louisiana during the summer, metacercariae may survive for extended periods (Malek, 1980). Once ingested by a ruminant host, the metacercariae excysts releasing a juvenile fluke. Excystment is an active process that occurs in 2 phases: (1) the metacercariae becomes activated in the rumen and; (2) after passing into the small intestine and contacting bile, the juvenile fluke escapes through a small hole on the ventral surface of the cyst wall (Dixon, 1966). The juvenile fluke penetrates the wall of the small intestine, migrates through the peritoneal cavity over a week's time, and

then penetrates through the liver capsule. Juvenile flukes migrate through the hepatic parenchyma for about 6 to 8 weeks before entering the bile ducts where they mature. Egg production can begin as early as 8 weeks post-infection (de Leon, Quiñones, & Hillyer, 1981), however most infections do not become patent until after about 11 to 12 weeks (Ross, Todd, & Dow, 1966). Thus, completion of the entire parasite life cycle, from the time an egg is shed onto pasture until a newly infected animal reinfects the pasture with the next generation of fluke eggs, requires 16 to 24 weeks.

Biology of *Fossaria cubensis*

Since the population dynamics of the lymnaeid snail intermediate host is fundamental to fluke transmission, understanding the biology of the snail is also important. In the United States, *Fossaria cubensis* and *Fossaria bulimoides* are the intermediate hosts of primary importance (Malone, 1986), however several other lymnaeid snail species including *Pseudosuccinea columella* and *Fossaria modicella* may also serve as hosts (Krull, 1934). In Europe and throughout much of the rest of the temperate enzootic range of *F. hepatica*, *Lymnaea truncatula*, is the principal snail intermediate host. Several other lynmaeid snail species of similar morphology and biology to *Fossaria spp.* and *L. truncatula* also serve as intermediate hosts for *F. hepatica* in various regions of the world (Malek, 1980). These snails are small (<10mm), "right handed" snails

that can be recognized by the dextral whorl of the shell and are semi-aquatic or amphibious in nature, being found most often in shallow water during the cooler months and on wet mud during the warmer periods of the year. These snails prefer water that is medium in chlorides (60 ppm), medium in calcium (21 ppm) and has a pH of 7.5 to 8.5 (Batte & Swanson, 1951).

Lymnaeid snails have the ability to survive adverse times of the year by hibernation during the winter in cold climates or by a similar process called aestivation during hot and/or dry periods. At such times they burrow into the mud and enter into a state of reduced metabolic activity. Snails that survive the adverse times of the year via these processes, reemerge when environmental conditions improve (spring in cold climates, fall in warm climates) forming the beginnings of the next generation. If snails are infected with *F. hepatica* when they enter into the hibernation or aestivation period, the parasite will also cease development until the snail reemerges. These infected snails thus provide a limited means of seasonal carryover of fluke infection.

Lymnaeid snails are hermaphroditic, therefore the entire sexually mature population is capable of producing fertile eggs (Olsen, 1944). *Fossaria bulimoides* attain sexual maturity when the shell length reaches 4.5 mm, and under optimal temperature and nutritional conditions, snails can grow to this size as soon as 14 days after hatching. These snails can produce from 2 to 3 egg masses per day containing

on the average 17 eggs. Once snails begin to lay eggs they can continue to do so for 3 to 7 months, which is probably close to their entire life span under natural conditions. Four laboratory raised snails produced an average of 5,112 eggs each over their lifetimes (Olsen, 1944). These features allow for tremendous increases in the density of snail populations over relatively short periods of time when environmental conditions are favorable. Snails are also capable of colonizing new areas by passive lateral migration from runoff of high water following periods of heavy or prolonged rainfall. Under such flooded conditions, snails often rise to the top and cling to the underside of the water surface.

Clinical Signs and Pathology

There are many similarities between cattle and sheep in the pathophysiology of fascioliasis but there are also some important differences. Acute disease is much more common in sheep than cattle and results from the extensive damage to the hepatic parenchyma caused by the migrating juvenile flukes. Chronic disease on the other hand results from a combination of the partially resolved hepatic damage that follows the acute phase and the blood sucking activities of the adult flukes within the bile ducts. In both species, chronic fascioliasis is the more common form. Black disease (infectious necrotic hepatitis) and bacillary hemoglobinuria

due to *Clostridium novyi* and *C. hemolyticum*, respectively, also can be complications of fascioliasis. The distribution of these two diseases parallels the distribution of *F. hepatica* (Jubb & Kennedy, 1970).

In cattle, infection with liver flukes usually causes no clinical signs and in general can be considered a subclinical disease. However, where extremely high fluke burdens rapidly accumulate and/or nutritional stress or other concurrent disease complicate otherwise subclinical infections, outbreaks of acute or subacute bovine fascioliasis can occur (Malone, Smith, Loyacano, Hembry, & Brock, 1982b; Reid, Doyle, Armour, & Jennings, 1972; Ross & Dow, 1966). Clinical disease resulting from chronic infection is most frequently seen in cattle under 2 years old and is characterized by weight loss, anemia, hypoproteinemia, eosinophilia, general depression and occasionally death (Armour, 1975). Concurrent infections with *Ostertagia ostertagi* can complicate the clinical presentation.

During the parenchymal phase of fluke migration in cattle, tissue regeneration is minimal and fibrosis is marked (Ross et al., 1966). In response to the traumatic injury caused by the flukes, tracts of coagulative necrosis develop which result in a diffusely fibrotic hepatic parenchyma containing hemorrhagic streaks and foci. In the bile ducts, the adult flukes produce a mechanical irritation which causes cholangiohepatitis. This leads to dilation, thickening and extensive fibrosis of the duct wall resulting in stenosis and calcification. This

biliary response is believed to be at least partially responsible for the short life span (less than 1 year) of the liver fluke in cattle.

In sheep, fascioliasis can occur either as an acute or chronic disease depending upon the pattern and level of infection. Acute disease occurs when large numbers of flukes are acquired over a short period of time. Unlike the response in cattle, during the parenchymal phase of the juvenile fluke migration in sheep, liver reaction is partially regenerative, fibrosis is minimal and parenchymal destruction is extensive. When large numbers of parasites are present, a critical period develops in which extensive hemorrhage occurs, causing the death of the host from acute fascioliasis (Dow, Ross, & Todd, 1968). Clinical signs include anorexia, depression and weakness. Sudden death may occur within 48 h of the appearance of signs and deaths may continue for 2 to 3 weeks (Wescott & Foreyt, 1986).

Chronic fascioliasis in sheep presents with similar but usually more pronounced clinical symptoms as those seen in cattle. Some deaths may occur but most animals will survive, albeit, in poor condition. The biliary reaction to the fluke is minimal and ducts expand to contain the parasites. Although some fibrosis of the duct wall does occur, the duct wall remains fairly pliable and calcification is not a feature. In this biliary environment flukes can survive for prolonged periods (Dow et al., 1968).

Host Immunity

Sheep acquire very little immunity to infection with *F. hepatica* evidenced by the fact that sheep develop only minimal fibrosis and no calcification of their biliary ducts and flukes have been reported to survive for up to 11 years. As a result, liver fluke infections are persistent, additive and essentially unimpeded by host response. This is largely responsible for the extreme pathogenicity of *F. hepatica* in this species (Boray, 1969). Cattle on the other hand develop a moderate degree of resistance to this parasite. The life span of flukes in cattle is quite variable and related to the pattern and intensity of infection (Ross, 1968). In low level single experimental infections (200 metacercariae), 75% of flukes that became established were lost between the 5th and 21st month after infection. In high level experimental infections (2,500 to 15,000 metacercariae), the life span was reduced to 6 to 7 months. Where challenge infections were involved the life span was reduced to 4 to 5 months and an "acquired self cure" was observed in that there was a turnover of the adult population. Ross (1968) concluded that the life span of the parasite in the bile ducts of cattle is directly related to the number of parasites present at any one time and to the intensity of the reaction that they produce in the bile ducts.

Doyle (1971) also studied the immune response of cattle to infection with *F. hepatica* but in greater detail. He found

that calves eliminated 85% of flukes derived from an initial experimental infection of 750 metecercariae between 16 and 30 weeks after infection. Additionally, a high level of resistance was seen in challenge infections where only 16% of the fluke burden found in control calves was recovered from calves which had been reinfected. Of the 2 groups of calves that were administered a challenge infection, 1 group was first treated with flukicide to remove the initial infection and the other was left untreated. Although the total number of flukes recovered from these 2 groups was similar, in the untreated group the flukes were largely derived from the initial infection indicating that these calves had resisted the establishment of the new population more successfully than did the treated group. In both of these studies an "acquired self cure" was demonstrated beginning approximately 4 months following initial infection. However, results of the challenge experiments in these 2 studies contradict each other. Whereas Ross reported that a new population of flukes replaced the old following challenge, Doyle reported that the old flukes persisted and an acquired immunity prevented the new population from becoming established. It is likely that differences in experimental design and the age and breeds of calves used were responsible for these differences.

From a practical standpoint, the fact that cattle exhibit a partially protective immune response is far more important than the particular details and differences of these studies.

It is clear that a dynamic relationship exists between the host animal and the parasite. The interaction of factors such as the age of the host, the innate resistance of the host, the previous exposure of the host and the present level of parasite exposure will determine the level of immunity, the degree of parasite establishment and the pathologic impact of the infection. Older cattle with previous exposure will have a greater resistance to infection than young parasite naive calves. Additionally, flukes are gradually eliminated over time so that most flukes acquired during the major transmission period of one year will be lost prior to the same time the following year. Fluke burdens in cattle are therefore not cumulative as they are in sheep. These issues should be considered when designing control programs for cattle.

Economic Importance

Economic losses from *Fasciola hepatica* result directly from increased death losses and liver condemnations at slaughter and indirectly from decreased livestock productivity. Although direct losses are easier to measure, indirect losses are considered to be far more economically important (American Association of Veterinary Parasitologists, 1983). Beef producers are affected by increased culling of cows, reduced sale weights of culled cows, lowered reproductive performance in the brood cow herd, and reduced calf weaning weights.

Economic losses in feedlots result from reduced feed-conversion ratios and lowered average daily gains, and fluke-infected dairy cows produce less milk (Hope Cawdery, Strickland, Conway, & Crowe, 1977; Randell & Bradley 1980; Malone, 1982; Simpson, Kunkle, Courtney, & Shearer, 1985).

Precise economic benefits of liver fluke control are difficult to quantify due to the interactions of physiologic, nutritional, associated disease, and climatic/geographic factors (Simpson et al., 1985). The interaction of these factors causes tremendous variation in pasture infectivity and the physiologic consequences of infection from year to year and ranch to ranch. As a result, there is little consistent and well-documented data on the benefits of liver fluke control. However, by piecing together data from several studies it is clear that the economic benefits of controlling liver flukes are substantial.

Significant production losses may occur in cattle herds when the prevalence of infection is greater than 25% (Malone, 1986). Subclinical infections averaging 54 flukes per calf (8 to 9 months old at the time of infection) reduced weight gains by 8% over the first 6 months of infection. Higher levels of infection (average of 140 flukes per calf, 14 to 15 months old) reduced weight gain by 28% and caused the appearance of clinical signs in some animals. Although impaired weight gains were demonstrated for up to 6 months, the majority of losses occurred during the first 4 months. Significant

production losses did not occur in infections of greater than 6 months duration (Hope Cawdery et al., 1977). In cow-calf studies in Louisiana, calves from groups of cows that were treated for liver flukes approximately 6 and 12 months earlier had a 10.1 kg advantage in 205-day adjusted weaning weight as compared to calves from groups of untreated cows. Additionally, several studies on the performance of fluke infected feedlot calves demonstrated that average daily gains were increased by an average of 9.5% by flukicidal treatment (Malone et al., 1982a). Dairy cows treated for liver flukes had a significant increase in average daily milk yield (+ 4.2 kg/day, 90-120 days after treatment) over daily yields of the previous lactation. Nontreated control and uninfected treated cows did not show significant gains over yields of the previous lactation (Randell & Bradley, 1980).

Data obtained from a large survey of cattle producers in Florida recently showed that specific benefits from control of liver flukes through appropriate treatment of cattle included 18-22 lb. heavier cull cows, 1-3 percent more calves, and 30-45 lb. heavier calves at weaning, yielding a net return to the producer of $15.19-$31.03 per brood cow, depending upon the size of the calf crop and calf prices (Simpson & Courtney, 1990). Based upon liver condemnations at slaughter, approximately 64% of beef cows in peninsular Florida are infected with liver flukes, thus proper fluke control applied to these one million plus beef cows has the potential to increase net

income to the cattle industry in Florida by 10 to 20 million dollars annually. A separate economic analysis of liver fluke control in Florida reported benefit-cost ratios ranging from 4:1 to 16:1 depending upon the number of treatments given and the range of estimates included in the analysis of production parameters (Simpson et al., 1985). A more recent study performed in central Florida on a large cow-calf herd reported that treatment with clorsulon showed a positive net return regardless of the price of calves and cows (Simpson, Greiner, & Richey, 1989). Cows treated with clorsulon had a net positive return that ranged from $46.96 for calf and cow prices of $0.60 and $0.35 per pound respectively, to $72.84 for calf and cow prices of $0.90 and $0.50 per pound respectively. Although Florida may have a more serious problem with liver flukes than most other states, these figures give an estimation of the economic impact which liver flukes can have on the cattle industry in highly enzootic areas.

Diagnosis

Fecal sedimentation remains the standard method for the diagnosis of fluke infections in individual animals. However, this technique is labor intensive (requiring 20 to 30 minutes per sample) and it can have poor sensitivity. Problems with sensitivity are due to: (1) the low egg output which is typical of fluke infected adult cattle; and (2) excessive

fecal debris in samples which may obscure the eggs and prevent detection. A modification of the sedimentation technique (Flukefinder®, Visual Difference, Moscow, ID) considerably improves both the sample processing speed and the sensitivity of egg detection. The technique utilizes a two sieve system which filters most of the fecal debris leaving a relatively clean sample which can then be sedimented and read. When examining fecal samples it is important to differentiate eggs of liver flukes (*F. hepatica*) from those of rumen flukes (*Paramphistomum spp.*). Rumen flukes are not affected by available flukicides and therefore eggs of these flukes are frequently present in greater numbers than those of liver flukes. Infections with the deer fluke, *Fascioloides magna*, do not become patent in cattle, and therefore cannot be diagnosed by fecal examination.

Several factors need to be recognized when interpreting fecal sedimentation results. As occurs with most parasitic diseases, flukes are over-dispersed within the cattle population, resulting in a situation where a small percentage of animals will carry the greatest fluke burdens and therefore shed the most eggs. This causes a large variation in the number of eggs shed by different animals grazing the same pasture. Additionally, since most fluke infected cattle shed relatively few eggs (less than 5 per gram even in heavily infected herds) (Malone & Craig, 1990), a minimum of 10 samples should be examined before clinical impressions of herd

prevalence are made. Other factors that must be considered are the long prepatent period of liver flukes and the variation in seasonal egg production that occurs depending upon transmission patterns and the duration of the infection.

Enzyme-linked immunosorbent assays (ELISA) have been developed for the serologic diagnosis of liver flukes, but none are commercially available presently. Diagnostic laboratories at veterinary colleges in Texas, Louisiana, Oregon and Washington have used several versions of these tests on trial bases but none proved sensitive or specific enough to improve on diagnosis by fecal sedimentation in individual patients (Malone & Craig, 1990). Although these tests have not been reliable for determining the infection status of individual cattle, they have potential usefulness in screening groups of animals such as stocker calves arriving at feedlots or young cattle on pasture suspected of harboring immature infections. Improvements in these tests continue to occur so it is likely that new versions could become commercially available in the future.

Seasonal Transmission Dynamics of *Fasciola Hepatica* in the United States

The seasonal transmission profile for liver flukes in any given locale depends upon a series of interactions between the biology of the parasite and the environment. Within this framework of interactions are two essential requirements for

the completion of the fluke life-cycle: (1) adequate moisture to sustain populations of the semi-aquatic lymnaeid snail intermediate host for prolonged periods; and (2) temperatures above 10°C, since below this temperature both parasite and snail development ceases (Ollerenshaw & Rowlands, 1959). The upper limits of temperature in which the parasite and snail can survive has not been well established. Since conditions necessary for completion of the fluke life cycle occur at different times of the year in each climatic/geographic region, the seasonal transmission patterns will also be different.

Transmission of *F. hepatica* in the Gulf Coast states occurs primarily during the winter and spring. In Louisiana, most fluke transmission occurs between the months of February and July (Malone, Loyacano, Hugh-Jones, & Corkum, 1984/85). A very similar pattern probably occurs in Texas as well (Craig & Bell, 1978). In north central Florida, fluke transmission occurred almost exclusively between the months of December and June, with the peak months being February to April (Boyce & Courtney, 1990). In Louisiana, this transmission pattern is directly related to yearly temperature and precipitation patterns. Soil moisture recharge begins in the autumn and snail populations increase throughout the late autumn and winter. By February, infected snails are shedding large numbers of cercariae. So long as rainfall is sufficient to maintain surplus soil moisture, snails will remain and cattle

will become infected with flukes. Fluke transmission ceases, however, with the first sustained drought of the summer since snails die or aestivate and metacercariae are rapidly killed (Malone et al., 1984). Water deficits tend to persist throughout the summer and early autumn, therefore snails remain in estivation until the autumn rains begin, usually in November. Similar seasonal climatic patterns are probably responsible for the transmission profile seen in Texas (Olsen, 1947; Craig & Bell, 1978). Climatic parameters responsible for the transmission profile demonstrated for northern Florida (Boyce & Courtney, 1990) have not been determined.

In southern Idaho, where the cold climate limits the pasture grazing season from April to November, fluke transmission occurred from June to November (Hoover, Lincoln, Hall, & Wescott, 1984). This transmission profile is similar to that of northern Europe (Armour, 1975; Ross, 1977; Shaka & Nansen, 1979). Summers in Idaho are semi-arid, therefore flood irrigation at regular intervals is routinely performed. This practice provides sufficient moisture for transmission to occur and paradoxically, it is the shallow irrigation ditches which provide the best snail habitat. It is likely that other areas of the cold temperate northwest United States have a similar transmission profile, however there are no other published reports to confirm this. More importantly, there are no published reports on seasonal transmission from any of the warmer areas of the western United States.

Rationale for Seasonal Control

To develop a rational seasonal control program for liver flukes in a given area, it is important to understand local transmission dynamics, fluke biology, seasonal pasture forage availability, local ranch management practices and the variable efficacy of available flukicidal drugs against the different age classes (juvenile, immature, mature) of *F. hepatica*. Currently, only two drugs are available in the United States for the treatment of liver flukes: clorsulon (Curatrem®, Ivomec-F®; Merck Sharp & Dohme Research Laboratories); and albendazole (Valbazen®; SmithKline Beecham Animal Health).

At the labeled dosage (7 mg/kg), Curatrem®, which is administered as an oral drench, has the best efficacy of these three products against adult stages (>99%) and will also kill immature bile duct stages (8-12 weeks) with fairly high efficacy (85 to 95%) (Kilgore, Williams, Benz, & Gross, 1985; Malone, Ramsey & Loyacano,1984; Malone, Williams, Lutz, Fagan, Jacocks, Jones, Marbury, & Willis, 1990). This product will only kill flukes, however, and has no effect on other parasites. Ivomec-F®, which is administered by subcutaneous injection, is a combination product which is formulated to deliver a full therapeutic dose of Ivomec® (200 μg/kg) plus a reduced dose (2 mg/kg) of clorsulon. The reduced subcutaneous dose of clorsulon in Ivomec-F has the approximate biological equivalence of a 3.5 mg/kg oral dose of Curatrem®. This dose

has about 97 to 99% efficacy against adult flukes (Kilgore et al., 1985; Wyckoff & Bradley, 1983; Zimmerman, Wallace, Schons, & Hoberg, 1986) but is not very effective against immature stages (Malone et al., 1984). The addition of Ivomec® makes this a broad spectrum product that will kill nematodes and arthropod parasites in addition to flukes. Valbazen® is a broad spectrum product, administered by oral drench, which kills flukes, nematodes and cestodes. At the labeled dosage (10 mg/kg), the efficacy of albendazole against adult flukes has been variable (76 to 92%) and the efficacy against immature stages is poor (Craig, Qureshi, Miller, Wade, & Rogers, 1992; Kilgore et al., 1985; Malone et al., 1982b). However, at higher doses (≥15 mg/kg), the efficacy of albendazole approaches that of clorsulon (Ronald, Craig, & Bell, 1979). None of these three flukicidal products will kill the migrating juvenile stage (<8 weeks), (Richards, Bowen, Essenwein, Steiger, & Buscher, 1990) therefore the limited efficacy of these drugs against juvenile and immature stages of *F. hepatica* must be taken into account when making decisions regarding the timing of treatments.

Flukicidal treatment is most effective if given when pastures are devoid of metacercariae and snails, and flukes infecting cattle are mature. This occurs during the late winter-early spring in northern climates and during the late summer-early autumn in southern climates. At these times virtually all the flukes in the pasture biota are concentrated

in the ruminant hosts as mature stages. Therefore, flukicidal treatment will not only kill most of the flukes in the cattle but will also reduce the potential for fluke transmission to snails when they emerge from hibernation or estivation.

Decisions regarding treatment of liver flukes must be made as part of an overall herd health management program and cannot realistically be done without accounting for other management factors such as stocking rate, pasture management and forage availability. Although animals will usually benefit from treatment regardless of other factors, treatment is most valuable in situations where cattle are also suffering from nutritional stress or some other production limiting disease. In the gulf coast states, most cattle are maintained year round on extensively managed open pastures. During the winter months, forage quality and availability is poor and most cows will lose substantial weight during this period even with supplementation. This is also the time of year when parasitic gastroenteritis can be an important health problem. Under these conditions fluke infections will have their greatest impact, thus treatment programs should aim to reduce fluke burdens during this time.

Current Treatment Recommendations for the Gulf Coast States

Fluke transmission in the gulf coast region ceases during summer because snail intermediate hosts burrow into the mud and aestivate, thus contamination of the pasture with

metacercariae ceases. Fluke control recommendations for beef cattle in the Gulf coast states are currently predicated on the occurrence of this summer gap in fluke transmission. Cattle on ranches having known endemic fluke infections should be treated with a flukicide once annually in the early autumn with an optional spring treatment based on local parameters of risk. The rationale behind this treatment scheme is as follows. Flukes are presumed to survive the summer only as adult flukes in the livers of cattle. Fluke eggs shed in the manure of cattle during the summer will be killed by the persistently high summer temperatures. Pastures will be cleansed of metacercariae shed in the spring by summer's heat and metacercariae are not replaced after snails enter summer aestivation. Finally, very few fluke infected snails are presumed to survive aestivation due to the combined stresses of both aestivation and fluke infection. Flukicidal treatment of cattle in the early autumn will prevent the shedding of large numbers of fluke eggs onto pasture when the essentially fluke-free snails emerge from summer aestivation with the onset of cooler weather. Since newly emerged snails and their subsequent offspring are not likely to become infected if cattle have been treated, fluke transmission to cattle is greatly reduced during the following winter and spring. As an added benefit, virtually all flukes in cattle in the autumn will be mature adults and therefore fully susceptible to flukicides.

The optional spring treatment is primarily palliative rather than curative, because many flukes would be immature at that time of year and thus not susceptible to flukicides. Its purpose is to reduce the fluke burdens carried over the summer by cattle during high risk years or on high risk properties (Malone, Williams, Muller, Geaghan, & Loyacano, 1987). Since some flukes will survive spring treatment, this treatment should be given in addition to, but never in place of an autumn treatment.

Current Treatment Recommendations for the Northwestern States

Fluke transmission in the northwestern U.S. ceases in December because temperatures are too low for parasite development and cattle are removed from pasture. Since most fluke transmission occurs from September to November, a single treatment should be given in the late winter or early spring. At this time all flukes will be mature and fully susceptible to flukicide treatment. By eliminating flukes from the cattle at this time, contamination of the pasture with eggs will be minimized in the spring when cattle are returned to pasture and snail populations return. Few infected snails, metacercariae, or eggs will survive the winter and do not seem to have an important epidemiological role in fluke transmission in this region. Autumn treatment is likely to be of little benefit because most flukes will be immature at this time and not susceptible to flukicides. Therefore, a single

annual treatment should never be given only in the autumn. Although a twice yearly (early spring/late autumn) treatment program could be used, because the fluke transmission cycle in cold climates is relatively short, a single late winter-early spring treatment should be satisfactory in most cases.

Epidemiology and Control of Bovine Fascioliasis in Florida: Review of Literature, Research Needs and Implications for Improved Control Recommendations

Swanson (1949) reported that the lymnaeid snails *Pseudosuccinea columella* Say and *Fossaria cubensis* Pfeiffer were the snail intermediate hosts of *F. hepatica* in Florida. These same snails were also reported to serve as intermediate hosts for the rumen flukes *Paramphistomum cervi* Schrank and *Cotylophoron cotylophorum* Fish which are commonly found in cattle raised in *F. hepatica* enzootic areas. In 1947 and 1948, economic losses resulting from condemned fluke-infected livers at the large slaughterhouses in Florida amounted to over $100,000. This value did not include losses of condemned livers in many small local butcher establishments throughout the state, nor did it include death or production losses (Swanson, Batte, & Dennis, 1952).

In 1952 liver fluke disease was known to be present in 25 of the 67 counties of the state. River tributaries, lakes and ponds were noted as constant sources of infection, especially if lime rock or marl-type soils were present. Seeping banks, springheads, bay heads and lands that were heavily limed also

were areas where snail intermediate hosts could be found. Cypress lowlands and piney woods areas were noted to be too acidic for snail development and these areas were therefore not important in fluke transmission (Swanson et al., 1952). Water from artesian wells was noted to create ideal breeding grounds for snails. These free-flowing wells also provide snails with areas of refuge where they can survive during periods of drought (Batte & Swanson, 1951).

Recommendations for control of fascioliasis were based on a program which included snail eradication through the use of pasture management and moluscicides, and treatment of cattle with hexachlorethane. Ranchers were advised to shut off artesian wells; drain low-lying wet areas using a V-type ditch cut; and where it was impractical to drain ponds, swamps or other wet areas, then those areas should be fenced off from cattle. Copper sulfate (bluestone, blue vitriol) was recommended as the moluscicide of choice to be applied at the rate of 20 pounds per acre if spread over pasture or 24 pounds per cubic foot per second of water flow if treating streams. After establishing a program of snail eradication, ranchers were advised to treat all cattle over 4 months of age with hexachlorethane at a dosage of 10 g/100 lbs. body weight. This treatment was to be repeated in 21 days to kill the flukes that were immature at the time of the first treatment and therefore unaffected by the drug (Swanson et al., 1952). Proper timing for this treatment or for the application of

moluscicide was not addressed. Apparently, no research into the seasonal transmission dynamics of liver flukes in Florida was performed and therefore the most beneficial time for performing these treatments was not known.

From June 1984 to June 1987, 37 groups of four fluke-free ewes each grazed in monthly succession a 0.25 ha pasture in order to determine the seasonal transmission dynamics of *F. hepatica* in north central Florida (Boyce & Courtney, 1990). The pasture, located on the University of Florida campus in Gainesville, was known to support populations of both *P. columella* and *F. cubensis*. At the end of each month, that month's group of ewes was held in a fluke-free pen for 2 months, necropsied, and the flukes recovered and counted.

Fluke transmission was found to occur from December to June with February, March and April as the peak months. This seasonal pattern was repeated with only minor variation over 3 years. No summer transmission occurred in any of the 3 years: in 1986 and 1987 transmission ceased in May and in 1984 and 1985 transmission ceased in June. Fluke transmission resumed during the late autumn or early winter: November (1986), December (1987), or January (1985).

This pattern of transmission was very similar to that reported in Louisiana and Texas (Craig & Bell, 1978; Malone et al., 1984/85), the other states of the Gulf coast where *F. hepatica* is highly enzootic. This gave the researchers confidence that this was the true transmission pattern for

north central Florida, although it was noted that data collected from a single study site may not be typical of an entire region. This may be particularly true for Florida, since northern Florida is in a warm temperate climatic zone whereas southern Florida is in a subtropical climatic zone. However, with no other data to base decisions on, veterinarians at the University of Florida have made fluke treatment and control recommendations for Florida cattle predicated on the absence of summer and early autumn transmission (Courtney, Shearer, & Plue, 1985; Shearer et al., 1986). It was acknowledged, however, that information on liver fluke transmission dynamics was desperately needed for southern Florida where 66% of Florida's beef cows are pastured.

Recently, a climate forecast model was developed for prediction of relative fluke risk in different years, and thus whether there is a need to treat cattle once or twice annually (Malone et al., 1987; Malone, Williams, Loyacano, & Muller, 1989). This forecast model also predicts the seasonality of fluke transmission for a given region. Although not fully verified for Florida, this forecast model correctly predicted in a retrospective study (Malone & Courtney, unpublished data) the transmission profile (i.e., the seasonality and intensity of fluke transmission) that had been reported by Boyce and Courtney (1990) in north central Florida. This same model, however, predicts that there may be two transmission seasons

(winter and summer) in subtropical southern Florida (Malone & Craig, 1990).

If fluke transmission in subtropical southern Florida occurs mostly in the winter and summer then treatment of cattle in early autumn and spring would be ill advised. Therefore, it is of extreme importance to the cattle industry of southern Florida to determine the seasonal transmission dynamics of *F. hepatica* in this region. Additionally, the seasonal transmission dynamics should be studied in central Florida to ascertain any differences that may exist in this transitional area.

The seasonal transmission dynamics of fascioliasis is often established for a particular area through the monthly slaughter of sentinel animals grazing known fluke-infected pastures, but this is an expensive process. Since transmission of *F. hepatica* closely follows the availability of its snail intermediate hosts, it is also possible to measure seasonal transmission of *F. hepatica* indirectly by studying the population dynamics these snails. The latter approach was taken in this project because it has the advantage of allowing the investigation of more sites at a far lower cost than can be done using sentinel animals. In addition to studying snail population dynamics, a DNA probe assay was developed to determine the infection prevalence of snail intermediate hosts with larval stages of *F. hepatica*. To determine whether runoff from an artesian well would alter the snail population

dynamics as compared to other snail habitats, one pasture was selected for study which had a free-flowing artesian well. Fluke egg survival during the summer was also studied, since summer transmission could only occur if eggs were able to survive and hatch during periods of extreme heat.

CHAPTER 2
SURVIVAL, DEVELOPMENT AND HATCHING OF EGGS OF *FASCIOLA HEPATICA* ON PASTURE DURING THE SUMMER IN FLORIDA

Introduction

Climate is the primary factor affecting seasonality of transmission for *F. hepatica* (Ollerenshaw & Rowlands, 1959). In cool-temperate areas of the world such as the northwestern United States, Ireland and Great Britain, liver fluke transmission occurs mostly during the summer and autumn (Armour, 1975; Hoover et al., 1984; Ross, 1977), whereas in warmer areas such as the Gulf coast region of the United States, transmission occurs primarily during the winter and spring (Boyce & Courtney, 1990; Craig & Bell, 1978; Malone et al., 1982a). Liver flukes in cool and warm climates will therefore face disparate environmental pressures for survival and successful transmission and natural selection will favor those parasites that successfully adapt to the set of environmental parameters which they face. Therefore, it is possible that data on transmission dynamics and bionomics of *F. hepatica* from a cool temperate climate may not accurately reflect the transmission dynamics and bionomics that exist in a tropical or subtropical climate.

Rowcliffe and Ollerenshaw (1960), determined the conditions necessary for the survival, development and hatching of eggs of *F. hepatica* in a series of experiments using eggs recovered from fluke-infected sheep in Great Britain. Great Britain has a cool temperate climate with maximum temperatures rarely exceeding 25°C. On the contrary, the areas of Florida where the majority of cattle are pastured and where *F. hepatica* is highly endemic is subtropical. In this region, daily high temperatures in the summer months average 33 to 34°C, with daily mean temperatures of 27 to 28°C. Rowcliffe and Ollerenshaw (1960) found that when eggs were stored in water free of fecal matter above 30°C, temperature increasingly inhibited development and mortality reached 100% when eggs were stored at 37°C for 24 days. In experiments where eggs were incubated in the presence of sheep feces at 100% humidity at temperatures of 18 or 27°C, mortality reached 100% by 24 and 13 days respectively. Eggs in sheep feces exposed to normal fluctuations of rain and drying during the summer reached greater then 90% mortality within 17 days and 100% mortality by 35 days.

Internal temperatures of cattle fecal pats deposited on open areas of pasture rise and fall in a diurnal pattern in accordance with air temperature and amount of solar radiation. In the early morning, air and fecal pat temperatures are the same, however, by mid-afternoon, fecal pat temperatures exceed air temperatures. In pilot studies, we frequently recorded

fecal pat temperatures in mid-afternoon of greater than 37°C with temperatures exceeding 40°C on some days. Although these high temperatures were not sustained for long periods, clearly the eggs in these fecal pats would frequently experience very hot temperatures (>35°C) for several hours and moderately warm temperatures (>27°C) for long periods of each day.

If eggs of *F. hepatica* in Florida have similar temperature limitations for survival and development as those in Great Britain, it seems highly unlikely that eggs on pasture would survive long enough to develop and hatch during the summer. However, if eggs of *F. hepatica* have adapted to the hot climate and can survive and hatch during the summer, then this would have important epidemiological implications. We have determined that the snail intermediate host for *F. hepatica* in Florida, *Fossaria cubensis*, is rarely present on pasture during the summer months in Florida. A dry period in the late spring (April and May) of most years usually causes snail habitats to dry and snails to aestivate. Snail populations do not reappear until the autumn even though snail habitats become wet once again when summer rains begin in June.

In most years, therefore, liver fluke transmission ceases during the in Florida summer because no new metacercariae are shed onto pasture once snails aestivate and metacercariae are rapidly killed under hot and dry conditions (Olsen, 1947). Fluke control recommendations for beef cattle in the Gulf coast states are currently predicated on the occurrence of

this summer gap in fluke transmission (Kaplan, 1994; Malone et al., 1982a). Flukicidal treatment of cattle in the early autumn will kill virtually all of the adult flukes thus preventing the shedding of large numbers of fluke eggs onto pasture when snails emerge from summer aestivation with the onset of cooler weather. Since newly emerged snails and their subsequent offspring are not likely to become infected if cattle have been treated, fluke transmission to cattle is greatly reduced during the following winter and spring. If eggs can survive on pasture for any length of time, however, flukicide would need to be given far enough in advance of the return of snails to gain the full benefit of this treatment. Current recommendations regarding the timing of autumn treatment do not take the possibility of egg refugia on pasture into account.

Alternatively, we have observed instances where small numbers of snails were present throughout the summer if snail habitats remained wet during the late spring and summer. Therefore in years with above average spring rainfall and normal or above average summer rainfall, it is possible that snail populations would be present on pastures in sufficient numbers to transmit liver flukes provided eggs survive long enough to infect them. Therefore, as part of a larger study to determine the seasonal transmission dynamics of *F. hepatica* in Florida it seemed desirable to determine the ability of

eggs to survive, develop and hatch on pasture during the summer months.

Materials and Methods

Collection of eggs. Eggs of *F. hepatica* were recovered from the bile of gall bladders collected from infected bovine livers at a local abattoir. Eggs were cleaned by rinsing the bile through two screens using tap water. The first screen (No. 60, 250μ mesh) kept large particulate matter from passage while allowing eggs of *F. hepatica* to pass through. The second screen (No. 400, 38μ mesh) allowed passage of clean bile, while trapping the eggs. The eggs were then stored in tap water at 4°C in darkness to inhibit development of the eggs.

Placement of egg-containing fecal pats on pasture. At the University of Florida's Animal Research Facility, Gainesville Florida, a section of unshaded pasture was mowed and a 0.3 m x 1.5 m x 3.0 m protective barrier of wooden stakes and translucent sheet plastic was constructed to protect the experiment from disruption. The barrier was sectioned in half, leaving two 1.5 m^2 plots. On 2 separate occasions (experiment 1, 21 June; experiment 2, 09 August) fresh feces was obtained from fluke-free calves at Sandhill Research Unit, University of Florida, Gainesville Florida. Six kg of feces was thoroughly mixed with the eggs of *F. hepatica* to a concentration of 100 eggs per gram of feces. The feces was

then divided into 2 even portions and replicate fecal pats were prepared by dropping the feces from a height of 1.25 m into each of the 1.5 m^2 plots. A sample of the feces was retained as a control to confirm the concentration of eggs in the feces and to determine egg development time and viability under controlled conditions.

Collection and processing of samples. A 4-gram sample was taken from each of the 2 fecal pats on: days 0, 1, 2, 4, 7, 15, 21, 28 and 35 in experiment 1; and days 0, 3, 7, 14, 21, 29 and 35 in experiment 2. Samples were collected during the mid-afternoon in the warmest portion of the day. All samples were taken from a moist portion of the interior of the fecal pat after a portion of the outer crust was removed. Following sampling, the crust was replaced to reform the pat and the sample was taken to the laboratory for processing. Sampling of fecal pats was ended when feces could no longer be collected due to disintegration of the pat. Two gram samples were processed using a modified sedimentation technique (Flukefinder®, Visual Difference, Moscow,ID). After filtering samples through the Flukefinder®, they were sedimented in 50 ml plastic conical test tubes for 3 minute intervals until the sample was clean. In most cases 2 to 3 sedimentations per sample was adequate. In samples with substantially more debris, an additional sedimentation was performed in a 15 ml test tube for 2 minutes. The concentrated suspension of eggs

was then transferred by pipette into 4 wells of a 24-well tissue culture plate and placed in darkness at 27°C.

Examination of eggs. Eggs were examined under an inverted microscope at weekly or biweekly intervals until fully developed miracidia that were near the hatching stage were noted. Thereafter eggs were examined daily. Prior to examination, plates were placed under artificial light for at least five minutes to induce hatching of mature miracidia. Daily examination was continued until at least 50% of all eggs had hatched or greater than 50% of eggs were dead. A classification scheme was established to follow the progressive development of the eggs as follows: undeveloped, developed, and hatched. An undeveloped egg had an interior that was completely filled with cells, appeared dense and granular and was golden brown in color. A developed egg had several stages including Stage 1+, Dead, and Stage 2+. Stage 1+ eggs lost their granular appearance and appeared coarsely cellular with or without empty space within the egg. A Dead egg was one that appeared either dead or dying; these eggs looked relatively acellular and were somewhat lighter in color. A Stage 2+ egg contained a formed miracidia. A hatched egg was empty and had an open operculum. All eggs present in the sample up to 100 were classified. The day of hatching was defined as that on which fifty percent of viable eggs had hatched (Rowcliffe & Ollerenshaw, 1960). Duplicate samples collected from the 2 fecal pats were processed and

examined separately. Results are reported as an average of the values obtained from the 2 replicate samples.

Weather information. Rainfall and temperature data were obtained from a local weather station at the Tobacco Unit, University of Florida, Gainesville, Florida. Additionally, during a pilot experiment (07-Jun to 18-Jun), daily air temperatures and fecal pat temperatures were recorded in the morning, early afternoon, and evening. Throughout experiments 1 and 2, air and fecal pat temperatures were recorded only at the time of sample collection.

Results

Experiment 1 (21 June to 26 July). Eggs from a control sample unexposed to pasture (day 0) required 15 days at 27°C to reach 50% hatching. Eggs from samples collected on days 1 and 2 hatched in the same amount of time as the control (14.5 and 15 days respectively), however, egg development and hatching was inhibited in samples taken on days 4,7, and 15 (Figure 2-1). Eggs from feces exposed to pasture for 4 days required 21.5 days to hatch whereas eggs exposed for 7 and 15 days required 18 and 20 days respectively. Eggs from samples collected on days 21 and 28 required less time in the incubator to hatch than the control eggs (11 and 10 days respectively), indicating that some degree of development had occurred within the fecal pat. Regardless of developmental inhibition or enhancement within the fecal pat, the longer the

eggs were contained within the fecal pat on pasture, the longer the total time needed for the eggs to hatch (time on pasture plus time at 27°C) (Figure 2-2). Throughout the sampling period egg mortality was minimal with greater than 97% of eggs still viable after 21 days on pasture and 80% still viable after 28 days. By day 35 the fecal pat was fairly well disintegrated from rainfall and the activities of dung disrupting invetebrates and an adequate sample could not be obtained.

During the first 2 weeks of this experiment (21 June to 04 July) there was a considerable amount of rain that helped keep the interior of the fecal pats wet (Figure 2-3, Table 2-1). Heavy and frequent rainfall continued over the next 3 weeks of the study causing fecal pats to disintegrate to a point where an adequate sample of feces could not be obtained by day 35. Fecal pat temperatures recorded at the time of sampling were 5°C to 10°C (average = 6.6°C) higher than air temperatures recorded at the same time and frequently reached 40°C or more (Table 2-2).

Experiment 2 (09 August to 13 September). Eggs from a control sample unexposed to pasture (day 0) required 16 days in the incubator at 27°C to reach 50% hatching. Eggs from samples collected on day 3 also required 16 days to hatch. Beginning with samples collected on day 7, the time required for eggs to hatch when transferred to 27°C decreased with each successive sample indicating that the eggs were developing

within the fecal pat (Figure 2-1). On the final sampling (day 35), greater than 50% of eggs recovered had already hatched by the time the eggs were first examined after being isolated from the feces. Although there was continuous development within the fecal pat, the total length of time required for the eggs to hatch increased with longer pasture exposure time (Figure 2-2). Throughout the sampling period egg mortality was minimal with 87% of eggs still viable after 21 days on pasture and 84% still viable after 35 days.

Only a small amount of rain fell during the first 2 weeks of experiment 2 (09 August to 13 September), however, the interior of fecal pats remained moist (Figure 2-3, Table 2-1). The frequency of rainfall increased over the next 3 weeks of the study which helped maintain adequate fecal moisture in the interior of the pat. Fecal pats disintegrated slower than in experiment 1 and adequate fecal samples were still obtained on day 35. However, by day 35 the integrity of the pat was severely disturbed and no further samples were taken. Fecal pat temperatures were 0 to 7°C (average = 2.8°C) higher than air temperatures recorded at the same time and on one occasion reached 41°C (Table 2-2).

Discussion

Our results on survival, development and hatching of eggs of *F. hepatica* exposed to summer environmental conditions differ substantially from those of Rowcliffe and Ollerenshaw

(1960). In studies on the bionomics of eggs of *F. hepatica*, they demonstrated that eggs will not develop in sheep feces or in concentrated fecal suspensions and development is inhibited by high temperatures (>30°C). Although both of these phenomena were demonstrated to some extent in experiment 1, in both experiments our data also contradict those findings. In our study, eggs of *F. hepatica* contained within feces for extended periods of time were able to survive and develop even though temperatures in the fecal pats frequently exceeded 40°C.

After being deposited onto pasture, fecal pats of cattle quickly form a dry outer crust helping to maintain a moist interior. In these experiments, the interior of the fecal pats remained moist for the duration of each experiment when disintegration of the pat finally occurred between days 28 to 35. In experiment 1, inhibition of egg development was observed in eggs from feces collected on days 4, 7 and 14, however, in experiment 2 inhibition was not observed. Apparently some combination of factors that inhibited development were present in the first experiment, but not the second. Based on the findings of Rowcliffe & Ollerenshaw (1960), one might expect higher temperatures early in experiment 1 to cause this difference. However, during the first 14 days of experiment 1 (21-Jun to 04-Jul), the average of mean daily and high temperatures were lower than those during the first 14 days of experiment 2 (09-Aug to 13-Sep)

(Table 2-2). Whether or not this small difference in air temperatures is important is debatable, however, since it is fecal pat and not air temperatures that will affect the eggs. Probably more important than air temperature is the amount of daily solar radiation. Although solar radiation was not measured in this study, average monthly values for solar radiation (available from published tables; ASCE, 1990) are greatest during June and July. The mean of average solar radiation values for June and July in Gainesville is 23,575 cal/cm^2, whereas the mean of average solar radiation values for August and September is 20,474 cal/cm^2. This difference may be important since fecal pat temperatures averaged 6.6°C higher than air temperature in experiment 1 whereas the difference in experiment 2 averaged only 2.8°C.

Differences in rainfall also may have contributed to the differences in egg development seen in these 2 experiments (Table 2-2). During the first 10 days of experiment 1 there were 9 days in which there was ≥ 0.1 cm of rain and total rainfall was 12.2 cm. The frequent and heavy rainfall during this period kept the fecal pats very wet. During the first 10 days of experiment 2 there were only 2 days in which there was ≥ 0.1 cm of rain and total rainfall was 0.6 cm. Although the interior of the fecal pat remained moist during this time, the fecal pats were noticeably dryer than in experiment 1.

We hypothesize that the differences in egg development seen between the 2 experiments are due to differences both in

fecal pat temperature and moisture content. Physical properties of a wet fecal pat are very similar to those of a concentrated fecal suspension, therefore it can be expected that egg development will not occur. However, when the outer crust of a fecal pat becomes dry it begins to crack and crumble allowing the interior to become increasingly aerated. Additionally, insect activity in and on the fecal pats causes numerous small hollows and cavities to form which further disrupts the integrity of the pat. Eggs would still be able to survive under the moist, high humidity conditions within the interior of the pat, but the increasingly oxygenated fecal environment would no longer resemble the more anaerobic environment of a concentrated fecal suspension. This would probably allow egg development to take place and would be a much different environment than that which exists in sheep feces. A combination of high fecal pat temperatures with a wet fecal suspension-like pat probably resulted in the inhibition of development that was observed in experiment 1. The dryer and cooler conditions of the fecal pat in experiment 2 enabled development to take place.

The maximum temperature that eggs could be exposed to and still survive was not determined. However, on 12 July a fecal pat temperature of 44°C was recorded and egg mortality was not increased over the previous collection. The threshold temperature for survival is therefore greater than 44°C, a temperature that would only occasionally be reached in fecal

pats on pasture. It is more likely that sustained high temperatures for long periods of time would kill the eggs, but this does not occur under natural conditions because of diurnal temperature variation. A pilot study was performed in early June for the purpose of optimizing the methods used in the actual study. The detailed results are not presented because of deficiencies in the methods and controls used in the pilot, but some of the results are noteworthy nonetheless. Most eggs from these fecal pats did not develop and egg mortality was very high after only 3 days on pasture (first collection). In one fecal pat no egg development was noted even after 41 days at 27°C. The other fecal pat had a few eggs hatch at day 14 (3%) and by day 41 only 14% had hatched with dead and undeveloped eggs accounting for 84% of the total. The period of time in which these fecal pats were on pasture was the hottest of the summer. During the 4 days that the fecal pats were on pasture (days 0-3), the average daily high temperature was 36.8 and the average daily mean temperature was 28.0°C with no rain and little cloud cover. Therefore, either the lethal threshold temperature is slightly greater than 44°C and that temperature was reached (44°C was the maximum fecal pat temperature recorded), or daily sustained temperatures in the fecal pats of >40°C for several hours were sufficient to kill most of the eggs. These results suggest that eggs deposited during a short, very hot period with day-long direct solar radiation will be killed. However,

because in Florida solar radiation reaches its greatest intensity in June and decreases thereafter, it is unlikely that fecal pats will reach temperatures sufficiently hot with high enough frequency to have a major impact on overall egg survival during the summer season.

Rowcliffe and Ollerenshaw (1960) determined that 9.5°C was the critical temperature below which egg development would cease. Using this value, they concluded that the thermal constant for the optimal temperature range for egg development was between 200 and 220 day degrees, the values obtained when eggs were incubated at 23 and 18 degrees respectively. Interpolation of graphs illustrating the number of degree days and the actual number of days required for egg hatching at different incubation temperatures (Rowcliffe & Ollerenshaw, 1960) gave a value of approximately 190 or approximately 11 days for 27°C. The eggs used in our experiments required 15 days to hatch when incubated at 27°C. Using the same critical temperature of 9.5°C, the number of day degrees necessary for hatching of the eggs used in these experiments is 263. Clearly, the eggs used in this study obtained from fluke-infected Florida cattle, developed much more slowly than the eggs obtained from fluke-infected sheep in Great Britain. Whether this difference in rate of development is due to a difference in the minimum critical temperature (which would affect the calculation of day degrees) or is due to an actual difference in development rate per degree above 9.5°C was not

determined in this study. It can be postulated, however, that the warm year-round temperatures of south-central Florida (average daily low temperature in January = 9.5°C, average daily mean temperature = 16°C) would minimize the selective pressure on eggs to develop at low temperatures and could therefore shift the thermal constant for the optimal temperature range upward.

The study of Rowcliffe and Ollerenshaw (1960) represents the only comprehensive examination of egg bionomics of *F. hepatica* that has been published. Our study was not meant to re-examine their results, but designed only to determine if eggs of *F. hepatica* could survive on pasture during the summer in Florida. The differences between the results of the 2 studies suggest that there is a distinct biological variation between liver flukes of Florida and Great Britain, but some of the differences also can be explained by dissimilarities in design. Sheep feces are deposited as small pellets and therefore will heat and desiccate much more rapidly than will cattle feces. Since cattle feces remain moist for long periods of time and become increasingly aerated as they dry and crack open, it is probably a much better environment for egg development than is sheep feces under drying conditions. However, the differences between the studies in egg development rate and survival at high temperatures suggest that the temperature requirements and limitations are different for *F. hepatica* in Florida than for *F. hepatica* in

Great Britain. Therefore, major differences in climate appear to have selected for parasites better able to survive under the subtropical conditions of Florida. It would be interesting to compare side by side using the same methods, the bionomics of eggs obtained from *F. hepatica* in a cool temperate climate (Great Britain, northwestern United States), a subtropical climate (Florida), and a tropical climate (Caribbean, Central America).

This study was performed under controlled conditions and only partially resembles the situation that would exist in the areas of a cattle pasture where fluke transmission is occurring. Eggs of *F. hepatica* require three essential factors to hatch. (1) They must be separated from the feces and later exposed to light, (2) the temperature must be adequate for survival, and (3) there must be a film of moisture on their surface during the entire developmental period from deposition onto pasture until release of the free-swimming, ciliated miracidia (Rowcliffe and Ollerenshaw, 1960). Furthermore, transmission of *F. hepatica* occurs only in and around the habitats of its snail intermediate hosts because only those eggs deposited in the wet snail habitat will ever be able to hatch, infect snails and ultimately infect their ruminant hosts. Eggs deposited elsewhere on pasture will almost never have the opportunity to infect a snail no matter how long the egg might survive. Also, water has a moderating effect on surface temperature so eggs

deposited in water will not be exposed to the same high temperatures as those deposited on dry ground. In a 2-year study of snail intermediate host bionomics in Florida where surface soil and water temperatures were recorded at 3-week intervals, surface soil and ground water temperatures reached 40°C only once, occasionally reached 37 to 38°C, and usually were less than 35°C. Taken together, the results of this study strongly suggest that eggs of *F. hepatica* can survive, hatch and potentially infect snails during the hot summer season in Florida. This conclusion has 2 important implications in regard to liver fluke epidemiology and control.

First, the timing of autumn treatment with flukicide must take into account the ability of eggs to survive on pasture during the late summer. Current recommendations for early autumn (September to October) treatment as the most advantageous time to treat cattle for flukes in Florida (Courtney et al., 1985; Kaplan, 1994; Shearer et al., 1986) does not take egg survival into consideration. This is especially important because fluke transmission in Florida is greatest during the spring months (Boyce & Courtney, 1990; Kaplan, 1994) and egg production peaks 16 to 20 weeks after infection (Doyle, 1971; de León et al, 1981). Therefore, flukes acquired during the spring will reach maximum egg production levels in the late summer. In Florida, emergence of snails from aestivation usually occurs in late September or

October, thus a large refugia of eggs will accumulate in snail habitats just before snails emerge from aestivation and begin to repopulate these areas. Treatment in late September or October is therefore too late to prevent pasture contamination with eggs capable of infecting the new generation of snails. The precise amount of time following treatment that is necessary for eggs previously deposited in snail habitats to hatch and die cannot be determined from the results of this study. However, it is likely that treatment approximately 4 weeks prior to the emergence of snails from aestivation should be sufficient time to allow eggs already on pasture to hatch and miracidia to die. Treatment for flukes in Florida should therefore be given in the late summer, mid-August to early September, to obtain maximum benefit.

The second important epidemiologic implication of egg survival during the summer is that in years when snail intermediate hosts are present on pasture during the summer, even in low numbers, it is very likely that they will become infected with liver flukes. Although the level of fluke transmission resulting from this situation will probably be low, flukes accumulated during the summer will not be killed with high efficacy by treatment with flukicide in the late summer or early fall because many flukes will still be immature and not fully susceptible to available flukicidal drugs. Surviving flukes will reach maximum egg output in the late autumn which is the time of year in Florida when snail

populations frequently reach their greatest levels. This would greatly decrease the benefits of late summer/early autumn treatment and would favor high levels of fluke transmission during the following winter and spring.

Table 2-1. Summary of precipitation and temperature data while fecal pats were on pasture.

	Experiment 1		Experiment 2	
	6/21-7/04	7/05-7/26	8/09-8/22	8/23-9/13
No. of rain days (≥ 0.1 cm/day)	9	11	5	13
Total rainfall (cm)	12.2	15.7	3.9	11.4
High temperature (°C)	36.6	37.6	37.5	36.4
Average of daily high temperature (°C)	34.2	35.3	35.4	33.6
Average of daily mean temperature (°C)	26.2	27.6	27.6	26.5

Table 2-2. Temperatures (°C) on sample collection dates, experiments 1 and 2.

Date	Weather station (daily high)	Pasture site*	Fecal pat*
Experiment 1			
22-Jun	35.3	33	38
23-Jun	35.1	33	40
25-Jun	34.9	33	40
28-Jun	31.7	30	35
06-Jul	37.6	35	42
12-Jul	35.6	34	44
19-Jul	35.8	ND	ND
26-Jul	36.5	34	39
Experiment 2			
12-Aug	36.2	34	41
16-Aug	34.8	32	35
23-Aug	36.4	32	35
30-Aug	33.0	33	33
07-Sep	33.9	29	31
13-Sep	33.2	33	36

* Temperatures recorded at time of sample collection in mid-afternoon
ND Not Determined

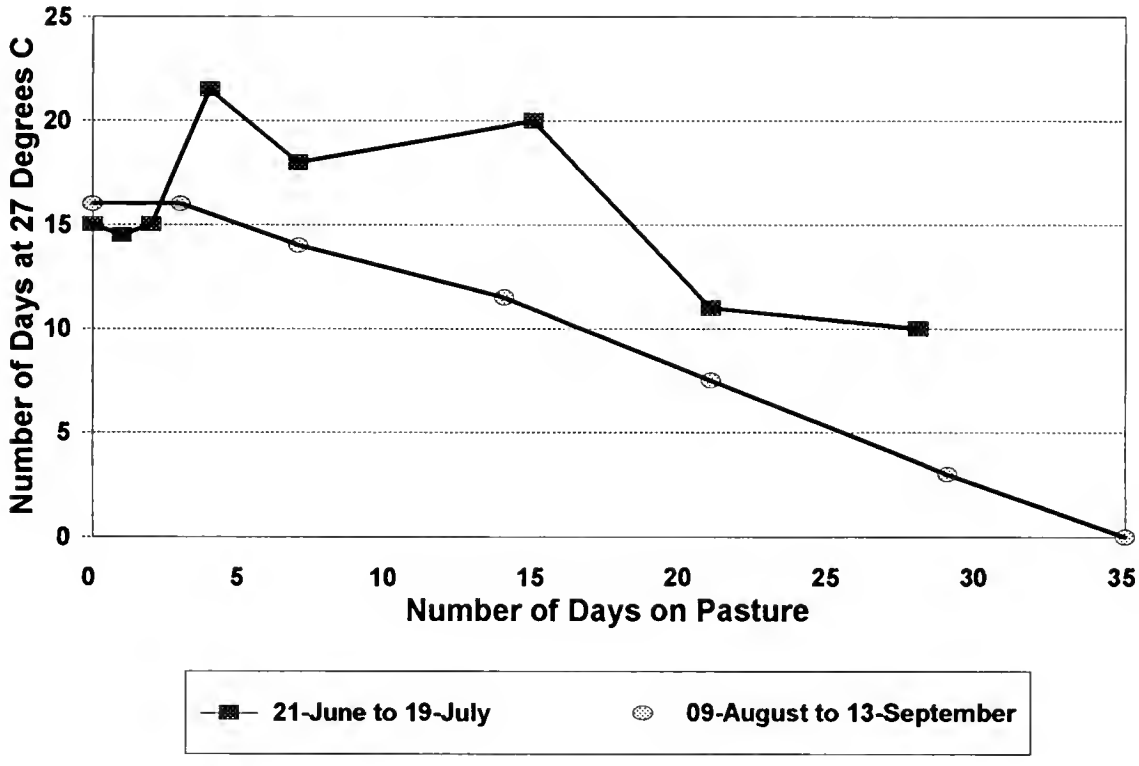

Figure 2-1. Number of days of incubation at 27°C required to reach 50% hatching vs. number of days that eggs were in fecal pats on pasture prior to collection.

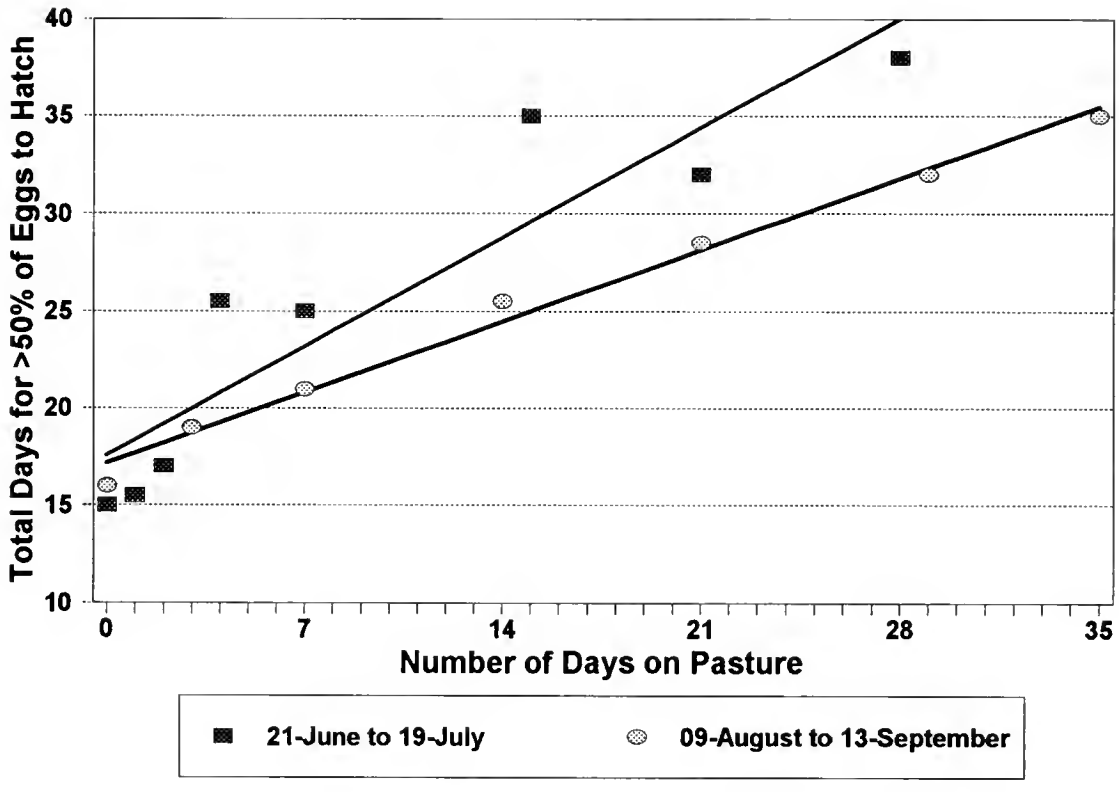

Figure 2-2. Total number of days (days on pasture plus days in incubator at 27°C) required for 50% of eggs to hatch vs. the total number of days that eggs were in fecal pats on pasture prior to collection. Linear regression was performed to obtain a best fit line for the data. The R^2 values for experiment 1 and experiment 2 were 0.85 and 0.99 respectively.

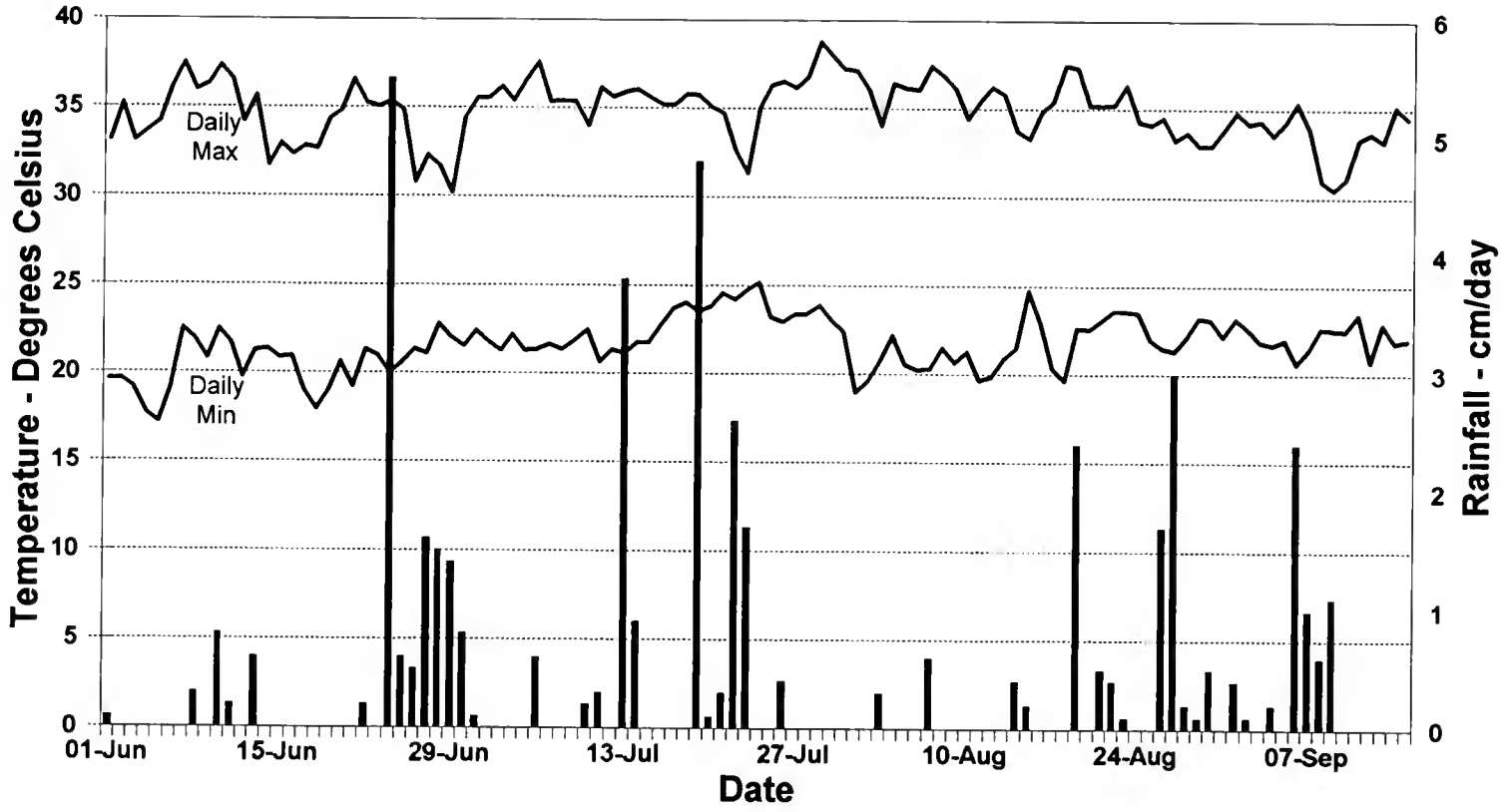

Figure 2-3. Temperature and rainfall data during summer 1993, Gainesville, Florida.

CHAPTER 3
A REPETITIVE DNA PROBE FOR THE SENSITIVE DETECTION OF *FASCIOLA HEPATICA* INFECTED SNAILS

Introduction

Fasciola hepatica, the common bile duct fluke of cattle and sheep has a worldwide distribution and is a major cause of liver condemnation, mortality and decreased productivity of livestock (Foreyt & Todd, 1976a; Malone et al., 1982a; Price, 1953). Transmission of *F. hepatica* is dependant on the presence of its lymnaeid snail intermediate hosts, thus data on the bionomics and infection rates of these snails can be used to evaluate changes in seasonal pasture contamination levels and the ensuing risk to grazing livestock. In the United States, *Pseudosuccinea columella*, *Fossaria cubensis* and *Fossaria bulimoides* are known to serve as the primary intermediate hosts for *F. hepatica*. However, *Fossaria* spp. are the more prevalent in enzootic areas and are the intermediate hosts of greatest importance (Malone, 1986).

Investigations into the epizootiology of *F. hepatica* frequently include the monitoring of snail infection rates. Historically, three techniques have been used to diagnose trematode infections of snails. These are (1) observation for cercarial shedding, (2) microscopic dissection, and (3)

crushing followed by microscopic examination. In the first method, individual snails are examined for the shedding of cercariae by placing them in glass tubes containing a small volume of water. This method has been used routinely in the study of schistosome infection rates in field populations of snails (Sturrock & Karamsadkar, 1979) but it has been used only rarely in published studies on snail infection rates of *F. hepatica* (Olsen, 1944).

In the second method, individual snails are carefully dissected under low and/or medium power microscopy. This technique has been used frequently for studying infection rates of snails with *F. hepatica* (Boray, Happich, & Andrews, 1969; Olsen, 1944; Smith, 1981), but there are several problems with it. First, snail dissection is tedious and time consuming. Second, intramolluscan stages of different trematodes are difficult to distinguish from one another prior to cercarial development, thus histological examination of stained tissue sections is often required to confirm the identification. The ruminant trematodes *Fascioloides magna* (giant liver fluke) and *Paramphistomum spp.* (rumen flukes of cattle and deer) share the same intermediate host and have the same enzootic range as *F. hepatica* (Castro-Trejo, Garcia-Vasquez, & Casildo-Nieto, 1990; Soulsby, 1982; Wescott & Foreyt, 1986) thereby confounding the identification of intramolluscan stages of trematodes recovered from snails. Third, prior to the release of rediae from the sporocyst and

their subsequent migration through the tissue of the snail (around day 21 post-infection), sensitivity of detection is poor even with careful dissection. Thus, dissection suffers from inadequate sensitivity in early stages of infection and in those few snails where early intramolluscan stages are found, specificity is problematic.

In the third method, snails are crushed and immediately examined under low power microscopy for the presence of rediae or cercariae. This technique has been used frequently in the study of snail infection rates with schistosomes (Chernin & Dunavan, 1962; Sturrock & Karamsadkar, 1979). A faster and less sensitive modification of this technique has been used recently in several large studies of the epizootiology of *F. hepatica* (Malone et al., 1984/85; Khallaayoune, Stromberg, Dakkak, & Malone, 1991). Although quick and simple, this modified technique fails to detect prepatent infections.

Because all three of these techniques have problems of sensitivity and/or specificity, the development of a highly sensitive and specific method for detecting infected snails would greatly aid in the study of the epizootiology of *F. hepatica* by enabling the collection of complete and accurate data on infection rates in field populations of snails. The use of a DNA probe capable of sensitive and specific detection of snails infected with *F. hepatica* could solve these problems. Highly repetitive DNA constitutes a large percentage of the eucaryotic genome (Britten & Kohne, 1986).

Since these sequences are present in multiple copies, the relative abundance of this repetitive DNA enables very sensitive detection. Additionally, these sequences evolve more rapidly than gene coding sequences making them excellent candidates for species-specific probes for eucaryotic organisms (McLaughlin, Collins, & Campbell, 1987). DNA probes using highly repetitive sequences have been developed for identification of several different parasite species: *Plasmodium falciparum*, (Barker, Suebsaeng, Rooney, Alecrim, Dourado & Wirth, 1986); *Brugia malayi*, (Sim, Piessens & Wirth, 1986); *Trichinella spiralis*, (Dame, Murrell, Worley, & Schad, 1987); *Onchocerca volvulus*, (Shah, Karam, Piessens, & Wirth, 1987); *Babesia equi*, (Posnett & Ambrosio, 1989); *Babesia bigemina*, (Buening, Barbet, Myler, Mahan, Nene, & McGuire, 1990); and *Opisthorchis viverrini* (Sirisinha, Chawengkirttikul, Sermswan, Amornpant, Mongkolsuk, & Panyim, 1991).

For a nucleic acid probe to be useful as a diagnostic tool in a large epidemiologic study, not only must the probe be highly sensitive and specific, but sample preparation must be relatively quick and efficient. Without efficient sample processing, a diagnostic probe cannot realistically be used in a study where thousands of samples need to be processed at a relatively low cost. We report here the development of both a DNA probe for the sensitive and specific detection of *F.*

hepatica infected snails and a quick and inexpensive DNA extraction protocol for use in field collected snails.

Materials and Methods

Trematodes and snails. Live adult *F. hepatica* obtained from condemned bovine livers at an abattoir in Florida, were incubated for 4 h in 0.85% NaCl at room temperature to remove adherent host cells and to empty intestinal cecae. Flukes were then rinsed once with DNA extraction buffer (50 mM Tris·HCl pH 8.0, 100 mM NaCl, 50 mM EDTA) and frozen with an equal volume of extraction buffer in an alcohol bath at -70°C. Adult *F. hepatica* from Montana also were obtained from condemned bovine livers, but these were frozen in liquid nitrogen following saline incubation. Eggs of *F. hepatica* were recovered from the bile of gall bladders collected from infected bovine livers and stored in the dark at 4°C. Rediae of *F. hepatica* were obtained by dissection of experimentally infected *P. columella* 28 days post-infection (p.i.), rinsed in DNA extraction buffer and quick frozen in 200 µl of DNA extraction buffer. Adults of *Fascioloides magna* and *Paramphistomum liorchis* were obtained from the liver and rumen respectively of white-tailed deer (*Odocoileus virginianus*) brought to a hunting check station in Florida. *Fascioloides magna* were incubated in 0.85% NaCl for 4 h and were quick frozen in liquid nitrogen. *Paramphistomum liorchis* were washed with 0.85% NaCl to remove contaminating rumen contents,

incubated in 0.85% NaCl for 4 h, rinsed once with DNA extraction buffer, and quick frozen with an equal volume of DNA extraction buffer in a liquid nitrogen bath. All trematodes were stored at -70°C until subsequent use in DNA preparation. *Pseudosuccinea columella* were obtained from established cultures in our laboratory (Boyce, Courtney & Thibideau, 1986) and *F. cubensis* were collected from a fenced field in Florida devoid of domestic ruminants.

Laboratory infections of snails. Eggs of *F. hepatica* were removed from the refrigerator and incubated in darkness at room temperature for two weeks to allow miracidial development. Following incubation, eggs were exposed to light to stimulate hatching. *Pseudosuccinea columella* or *F. cubensis* were individually placed in wells of a 24 well tissue culture plate along with 2 - 3 freshly hatched miracidia and 200 - 300 μl of tap water. After 3 - 4 h the snails were removed from the wells and reared in culture.

Isolation of genomic DNA. Frozen flukes were ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. Genomic DNA was extracted from the powdered flukes by SDS-Proteinase K digestion followed by phenol/chloroform extraction (Sambrook, Fritsch & Maniatis, 1989). The recovered DNA was dissolved in 10mM Tris·HCl-1mM EDTA, pH 7.6 (TE) buffer, and contaminating RNA was removed by incubation with RNAse A for 60 min at 37°C followed by a second phenol/chloroform extraction and ethanol precipitation.

Uninfected snails were kept without food in distilled water containing 100 μg ml^{-1} ampicillin for 48 h to clear their digestive system and minimize bacterial contamination prior to the extraction procedure (Strahan, Kane, & Rollinson, 1991), rinsed in distilled water, and their shells crushed and removed. Intact tissue from four snails was rinsed once in DNA extraction buffer, placed in a microcentrifuge tube with 400 μl of extraction buffer and crushed with a polypropylene pestle (Kontes Chemistry & Life Science Products, Langhorne PA). Genomic DNA from snails was then extracted essentially as described above.

Construction of *F. hepatica* genomic DNA library. Genomic DNA of *F. hepatica* was digested to completion with the restriction endonuclease Sau 3A (BRL, Gaithersburg MD), purified by phenol/chloroform extraction and recovered by ethanol precipitation. Digested DNA was ligated into the Bam H1 site of the plasmid Bluescript® SK+ (Stratagene, La Jolla, CA) using T4 DNA ligase. Ligation products were transformed into *Escherichia coli*, strain XL1-Blue and were grown overnight at 37°C on Luria Bertaini (LB) plates containing ampicillin, tetracycline, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropylthio-β-D-galactoside).

Selection of clones containing repetitive sequences. Ninety six recombinant clones were selected at random, transferred to a 96-well microplate and grown overnight in LB media at 37°C. Replicates of the clones were made on

nitrocellulose filters (Schleicher & Schuell, Keene, NH) placed on LB plates, grown overnight at 37°C, treated to release the DNA from *E. coli* (Thayer, 1979) and baked for 2 h in a vacuum oven at 80°C. The filters were incubated for 2 h at 65°C in a prehybridization solution (6X sodium chloride, sodium citrate pH 7.5 (SSC), 1.0 mM EDTA, 10X Denhardts, 0.5% SDS, 10 mM $NaPO_4$, and 100 $\mu g\ ml^{-1}$ denatured salmon sperm DNA). A radiolabeled probe was prepared using genomic DNA of *F. hepatica* and a random primer labeling kit (Prime-It®, Stratagene, La Jolla, CA) with α^{32}P-dATP (111 TBq/mMol, E. I.DuPont De Nemours & Co. Inc., Wilmington, DE). Labelled probe was boiled for 5 min with 1.0 mg of sheared salmon sperm DNA and added directly to the filters in prehybridization solution for overnight hybridization (16 h). The filters were washed once in 6X SSC, 0.1% SDS at room temperature for 10 min, twice in 2X SSC, 0.1% SDS at 50°C for 30 min, and once with 0.2% SSC, 0.1% SDS at 50°C for 30 min. Hybridizing clones were identified by autoradiography using X-OMAT-AR film (Kodak, Rochester, NY) and an intensifying screen. Positive clones were grown in LB broth at 37°C overnight, and plasmid DNA was isolated by the boiling prep method (Holmes & Quigley, 1981). Insert DNA was excised by digestion with Eco RI and Xba I and DNA fragments were separated by agarose gel electrophoresis. DNA fragments were transferred from the gel to a nylon membrane (Southern, 1975) and probed with radiolabeled genomic DNA of *F. hepatica* as described above.

Sensitivity and specificity of selected clones. Replicate slot blots were made onto nylon membrane (Hybond N+®, Amersham, Arlington Heights, IL) using a vacuum filtration apparatus (Minifold II Slot Blot System - Schleicher & Schuell, Keene, NH). Blots were made with 1.0 μg each of genomic DNA from adults of *F. magna*, *P. liorchis* and *F. cubensis*, and 1.0 μg, 100 ng, 10 ng, 1.0 ng, and 0.1 ng of genomic DNA from *F. hepatica*. DNA was crosslinked to the membranes by exposure to UV light (Stratalinker®, Stratagene, La Jolla CA) prior to prehybridization. Each of the selected clones was radiolabeled for use as a probe and was hybridized overnight with one of the replicated slot blots. Hybridization and washing conditions were the same as described for the genomic DNA library screen for clones with repetitive sequences.

Determination of fragment size and insert number in F. hepatica specific clones. Insert DNA of *F. hepatica* specific clones was PCR amplified in a Perkin Elmer Cetus DNA thermal cycler using a standard reaction mixture (AmpliTaq®, Perkin Elmer Cetus, Norwalk CT) with KS (GCTATGGCAGCTGGAGC) and SK (TCTAGAACTAGTGGATC) primers. PCR products were digested with Sau 3A for 1 h at 37°C and fragments were separated by electrophoresis on a 1.5% agarose gel.

Sensitivity and specificity of selected probe. Genomic DNA from Florida and Montana isolates of *F. hepatica*, genomic DNA of *F. magna* and *P. liorchis*, and genomic DNA from the

Florida isolate of *F. hepatica* mixed with 10 μg of *P. columella* DNA was spotted in 10X dilutions from 1.0 μg down to 10 pg onto nylon membrane. DNA of rediae dissected free of experimentally infected snails, and DNA of individual fluke-infected snails (28 days p.i.) also were spotted onto the membrane. Hybridization and washing conditions were the same as described for the genomic DNA library screen.

Minimum biologic sensitivity of the probe. Laboratory raised *P. columella* were infected with miracidia of *F. hepatica*. Groups of 4 snails were removed from culture tanks on days 0 (4 h post-infection), 1, 4, 7, 14, 21, and 28 p.i., crushed in 200 μl lysis buffer (8% triton-X 100, 250 mM sucrose, 50 mM TE pH 7.6) using plastic pestles, and frozen at -20°C prior to extraction. DNA was extracted using a protocol which was modified from that used by Strahan et al. (1991). Snail lysates were thawed, 20 μl of freshly prepared Proteinase K (10 mg ml^{-1}) was added, and samples were incubated in a waterbath at 68°C for 2 h. Following incubation, samples were centrifuged for 1 min at 12,000 x g and the supernatant was transferred to a clean tube. Nucleic acids in the lysate were precipitated by the addition of 800 μl of warm 2% hexadecyltrimethyl ammonium bromide solution (CTAB) and tubes were inverted 10 times to mix. The precipitate was pelleted by centrifugation for 1 min at 12,000 x g. Supernatant was aspirated and 200 μl of 2.5 M NaCl containing 10mM EDTA was added to dissolve the pellet. To facilitate dissolution the

samples were placed back into a 68°C waterbath over night. A single chloroform extraction was performed using 400 µl of chloroform/isoamyl alcohol (24:1) and the aqueous phase was transferred to a clean tube. Twenty µl of 2N NaOH was added to denature the DNA and the sample was applied onto nylon membrane using a dot blot manifold (Schleicher & Schuell, Keene, NH). DNA was crosslinked to the membrane using UV light and was hybridized with α^{32}P-dATP labeled probe overnight at 65°C as described above. The hybridized membrane was washed twice for 15 min in 2X SSC, 0.1% SDS and once in 0.2X SSC, 0.1% SDS for 15 min at 55°C.

Results

Screening of F. hepatica genomic library for highly repetitive sequences. Thirteen out of 96 recombinant clones from a genomic DNA library of *F. hepatica* hybridized strongly with radiolabeled genomic DNA of *F. hepatica*. All clones contained small inserts, ranging in size from approximately 190bp to 900bp. Ten of the 13 clones were selected for further analysis. All 10 clones displayed strong hybridization signals when probed with total genomic DNA of *F. hepatica*, suggesting that they contained highly repetitive sequences.

Characterization of selected clones. Radiolabelled probes were prepared from each of the 10 clones to identify those which hybridize specifically with *F. hepatica* DNA but

not with DNA of *F. cubensis*, *F. magna* or *P. liorchis*. Four clones cross hybridized with DNA of *F. magna* and one clone cross hybridized with DNA of *P. liorchis*. Most clones showed a slight non-specific cross hybridization with DNA of *F. cubensis*. This slight non-specific signal was not seen in later experiments with DNA of *F. cubensis* or *P. columella* confirming the specificity of these clones against DNA of the snail intermediate hosts. Each of the 5 clones specific for *F. hepatica* detected as little as 1.0 ng of *F. hepatica* genomic DNA (Figure 3-1).

Insert DNA of the 5 *F. hepatica* specific clones ranged in size from 190bp to 520bp. Inserts from these clones were amplified by PCR using vector derived primers (TATAGGGCGAATTGGGT, GGTGGCGGCCGCTCTAG) and were digested with Sau 3A. A 125bp fragment was seen in all 5 of the clones. Since all 5 clones contained a similar sized DNA fragment, cross-hybridization experiments were performed to determine the relationship of the 5 clones. Results (not shown) revealed that insert DNA of all 5 clones hybridized with varying intensity to probes prepared by radiolabelling each of the 5 cloned inserts.

pFh5 and pFh76 contained cloned inserts of approximately 250bp and were selected for sequencing since this suggested that each clone may contain two-125bp repeats. Clone pFh5 contained two 124bp repeats (pFh5r1 & pFh5r2) and clone pFh76 contained one 124bp repeat (pFh76r1) together with a short

unrelated sequence. The sequences of the 3 repeats differed only slightly from each other. Repeats pFh5r1 and pFh5r2 had 11 base mismatches, pFh5r1 and pFh76r1 had 14 base mismatches, and pFh5r2 and pFh76r1 had 15 base mismatches. Thus, the 3 repeats have a 91%, 89%, and an 88% sequence identity respectively (Figure 3-2). A search of the Genbank database showed that no other previously reported sequences were homologous to those reported here.

The abundance of this 124bp repeat within the *F. hepatica* genome was determined by quantitative dot-blot analysis (Figure 3-3). Phosphor image analysis of hybridization signals demonstrated that this 124bp repeat constitutes ~ 15% of the *F. hepatica* genome. Assuming that the genome of *F. hepatica* is similar in size to that of *Schistosoma mansoni*, i.e. 2.7×10^8 (Simpson, Sher & McCutchan, 1982) there are greater than 300,000 copies of this 124 bp repeat present in the genome of *F. hepatica*.

Sensitivity and specificity of the selected probe, pFh5. 1.0 ng of genomic DNA of *F. hepatica* was detected easily with the probe and this level of sensitivity was not diminished by the presence of 10 μg of snail DNA. The probe showed similar hybridization intensities to genomic DNA of the Montana and Florida isolates of *F. hepatica* verifying the reliability of the probe against another isolate from a geographically distant locale (data not shown). The probe did not cross hybridize with DNA of the trematodes *F. magna* or *P. liorchis*,

or with DNA of the snail intermediate hosts, *F. cubensis* and *P. columella*. Additionally, DNA from a field-collected snail (*F. cubensis*) infected with schistosome-like cercariae presumptively diagnosed as *Heterobilharzia americana* did not hybridize with pFh5. *Heterobilharzia americana* is the only other digenetic trematode other than *F. hepatica*, *F. magna* and *Paramphistomum spp.* known to use *Fossaria spp.* as intermediate hosts (Malek, 1980).

Since the purpose of this probe is to detect all infected snails, the goal set for sensitivity was the detection of the minimum biologic unit of the parasite. Thus, if a single miracidium following penetration of the snail can be detected, no further sensitivity is required. The probe developed in this study is capable of this level of sensitivity (Figure 3-4). This probe detected the parasite DNA in all 4 snails whose DNA was extracted less than 4 h following miracidial penetration. The amount of parasite DNA remained about the same on day 1 p.i., but by day 4 considerable parasite development had occurred and this development continued in a logarithmic fashion throughout the 28 day time period included in the experiment (Figure 3-5, Table 3-1). One infection was not detected on day 14, and this was most likely due to the snail never having become infected or the snail having rejected its infection, although laboratory error during the extraction and detection process is also possible. These snails were each exposed to 2-3 miracidia to ensure that most

snails would become infected, however, in a separate experiment, DNA from a single miracidia extracted together with an uninfected snail was easily detected with the probe (data not shown). Data from the phosphor image analysis of blots containing known dilutions of genomic DNA of *F. hepatica* and the same dilutions of DNA of *F. hepatica* but added to snails and extracted, demonstrated that DNA recoveries using the CTAB based extraction protocol average 50-60% regardless of the concentration of the added DNA of *F. hepatica*.

Discussion

The DNA probe and snail extraction protocol reported here solve the problems of sensitivity, specificity and efficiency which are inherent in the visual examination of snails for the purpose of detecting *F. hepatica* infection. Infected snails can be detected immediately following miracidial penetration, thus a sensitivity equivalent to the minimum biologic unit of the parasite is achieved. Additionally, signal intensity increases in a logarithmic fashion over time, therefore, signal intensity can be used to estimate the age of the infection giving further information on the bionomics of the infected snail populations on pasture. However, for a nucleic acid probe to be useful as a diagnostic tool, not only must it be sensitive, but it must also be specific for the organism of interest. The screening and selection process used in this study ensured that the probe would be both highly sensitive

and specific. pFh5 was selected from among the 5 candidate probes based upon sequencing results which showed that it contained two repetitive sequences but no other unrelated DNA. This probe did not cross hybridize with 1.0 μg of genomic DNA of *F. magna* or *P. liorchis*, trematodes which share the same enzootic range and same intermediate hosts as *F. hepatica*.

All 5 of the clones which were specific for *F. hepatica* contained a fragment of DNA approximately 124 bp in size. In experiments to determine if these fragments were related, all 5 cross-hybridized with each other to varying degrees suggesting that they all were similar but not identical in sequence. This conclusion was further supported when sequence data from clones pFh5 and pFh76 demonstrated that the 3 repeat sequences contained in these clones shared only about 90% identity. This level of sequence variation between repeats is characteristic of interspersed repetitive sequences (Jelinek & Schmid, 1982), although the arrangement of this repeat in the genome has not been determined. It therefore appears that the *F. hepatica* genome contains a large family of 124 bp repeats that are not identical but share a high level of sequence identity. Quantitative dot-blot analysis revealed that this 124 bp repeat accounts for approximately 15% of the entire *F. hepatica* genome. This level of abundance is similar to that reported for a 121 bp repeat (12%) in *S. mansoni* (Hamburger, Turetski, Kapeller & Deresiewicz, 1991). It is interesting that a search of the Genbank data base revealed no

homology between the 124 bp repeat sequences reported here and the 121 bp repeat sequence reported for *S. mansoni*. The 124 bp repeats of *F. hepatica* and the 121 bp repeat of *S. mansoni* were cloned from genomic DNA libraries made from DNA digested with Sau 3A. It would be interesting to see if other trematode species also contain abundant repeats of similar length as this would be an excellent means of quickly isolating species specific DNA probes.

Before a nucleic acid probe can be used in a large epidemiologic survey, sample processing must be made both time and cost efficient. Phenol/chloroform extraction was used in the development and initial testing of the probe but problems associated with this technique excluded it from practical consideration as a method for large scale snail DNA preparations. Hamburger, Weil, & Pollack (1987), reported a NaOH-based technique for quick DNA extraction of *Schistosoma mansoni* infected snails (*Biomphalaria glabrata*). However, in our laboratory using different snail species (*F. cubensis*, *P. columella*), poor results were obtained using this technique.

We have developed a DNA extraction protocol which solves all the major problems associated with both of these techniques. The protocol described here utilizes the cationic quaternary ammonium compound, CTAB, which selectively precipitates nucleic acids in preference to proteins and polysacharides at low salt concentrations (Yap & Thompson, 1987). DNA recoveries using this procedure average 50-60%,

and the extract is easily filterable through a nylon membrane. When extracting large numbers of snails with this technique, total processing time per sample, excluding an overnight incubation, is only about two minutes. Total cost per snail for the entire assay including all miscellaneous supplies, solutions, reagents, enzymes, and disposables (i.e. tubes, pipette tips, etc.) is only about US $0.33. This sample processing speed enables extraction of hundreds of snails over a 2 day period and the low cost minimizes economic considerations when determining the number of snails that must be assayed (thousands) to obtain statistically meaningful data. This protocol therefore possesses all of the desirable qualities of a DNA extraction process for use in a large scale epidemiologic study. It is quick, inexpensive, safe, and gives both excellent DNA recovery and an extract that is easily filterable.

Recently, nucleic acid probes for the detection of *F. hepatica* infected snails have been published in two separate reports (Shubkin, White, Abrahamsen, Rognlie, & Knapp, 1992; Heussler, Kaufman, Strahm, Liz, & Dobbelaere, 1993). Shubkin et al., (1992) reported the development of a nucleic acid probe using rRNA sequences for the detection of *F. hepatica* infected snails, however, the probe was not tested for specificity against any other trematodes. *Fascioloides magna* and *F. hepatica* both belong to the family Fasciolididae. Therefore, it is very likely that the DNA of these organisms

have a high degree of sequence homology, especially in conserved genes such as those for rRNA. In addition, *Paramphistomum spp.* and *F. magna* both can infect the same snail intermediate hosts as *F. hepatica* and frequently infect livestock in the same areas enzootic with *F. hepatica*. Thus, before any nucleic acid probe can be used as an epizootiologic tool in the study of *F. hepatica*, it must be demonstrated that it does not cross hybridize with nucleic acid of either of these trematodes. Additionally, this report did not address the question of sample preparation efficiency and cost, both of which will ultimately determine whether a nucleic acid probe becomes a useful research tool or an academic exercise.

Heussler et al. (1993) also failed to demonstrate the practical specificity of the DNA probes they developed. They screened their probe for specificity against 3 different trematodes: *Dicrocoelium dendriticum*, *Xiphidiocercaria spp.*, and *Diplostoma spp.* All of these trematodes are phylogenetically divergent from *F. hepatica*, use different snail intermediate hosts, are found in different ecological niches than *F. hepatica*, and only *D. dendriticum* is a parasite of ruminants. Additionally, sensitivity of these probes was not tested against purified genomic DNA of *F. hepatica* and when squash blots of individual *F. hepatica* rediae or cercariae were probed, only a weak signal was detected. This is in contrast to the sensitivity of the probe assay reported here in which the DNA of a single miracidia can be detected

and the DNA of a single rediae gave a very strong hybridization signal that was similar to the signal strength seen in 14-21 day old infections.

The DNA probe assay reported here will enable researchers to obtain truly accurate infection rate data in field-collected snails for the first time. This should lead to a better understanding of the epizootiology of *F. hepatica*. By monitoring the population density and infection rates of snails over time, patterns of seasonal pasture infectivity with metacercariae can be determined. Knowing this, the risk of infection to grazing ruminants can be predicted. Because numerous snail habitats in a given region can be studied at a relatively low cost using this approach, the chances of geographic bias are reduced greatly. This is extremely important for any disease in which transmission is dependant upon an invertebrate animal whose population density is so easily affected by minor differences in local physiographic parameters and precipitation levels.

Monitoring of schistosome infection rates in snails frequently has been used to evaluate the success of control measures and to better understand transmission dynamics (Barnish, 1982; Chernin & Dunaven, 1962; Christie & Upatham, 1977). This has not been attempted for *F. hepatica*, however. By monitoring snail infection rates with *F. hepatica*, the long term effects of currently recommended control measures on liver fluke transmission dynamics can be better evaluated.

This may permit the adjustment of fluke risk prediction models (Malone et al., 1987) to compensate for chemotherapeutic intervention, thus making improved control recommendations possible.

Additionally, this DNA probe assay can be used to increase our basic understanding of the biology of the snail-fluke relationship. This includes the ability to rapidly determine snail host suitability for *F. hepatica* in areas of the world where it is not yet known. Finally, it is very likely that the DNA probe assay reported here for the detection of *F. hepatica* infected snails can be modified and used for the study of other helminth parasites which utilize an invertebrate animal during a portion of their life-cycle.

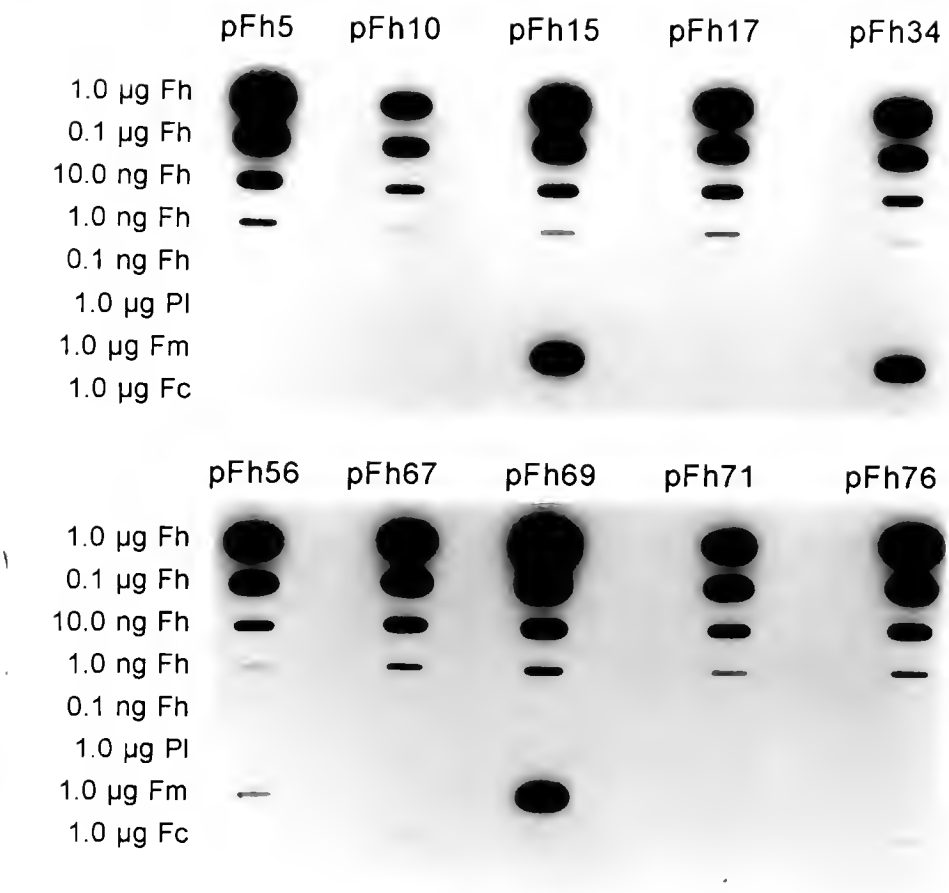

Figure 3-1. Screening of clones containing repetitive sequences for sensitivity and specificity. Each clone was radiolabeled with α^{32}P-dATP and used to probe replicate slot blots containing DNA of *F. hepatica* (Fh) in 10X dilutions from 1.0 µg down to 0.1 ng, and DNA of *F. magna* (Fm), *P. liorchis* (Pl) and *F.cubensis* (Fc) in 1.0 µg quantities.

```
          1                                                   50
pFh5r1    GATCAATTCA CCcATTTCtG tTAGTCCTAC TGGAATTTCT CTTGTgCCAA
pFh5r2    GATCAATTCA CCcATTTCcG tTAGTCCTAC TGGAATTTCT CTTGTgCCAA
pFh76r1   GATCAATTCA CCtATTTCcG cTAGTCCTAC TGGAATTTCT CTTGTaCCAA
Consensus GATCAATTCA CC-ATTTC-G -TAGTCCTAC TGGAATTTCT CTTGT-CCAA

          51                                                  100
pFh5r1    TGcGTTTCTC AGGCCGTGAc TAtCCTcTgG ACAAtgAATa AGCgTGCCtG
pFh5r2    TGcGTTTCTC AGGCCGTGAc TAtCCTcTgG ACAAacAATc AGCtTGCCcG
pFh76r1   TGtGTTTCTC AGGCCGTGAt TAcCCTaTtG ACAAagAATc AGCgTGCCcG
Consensus TG-GTTTCTC AGGCCGTGA- TA-CCT-T-G ACAA--AAT- AGC-TGCC-G

          101                   124
pFh5r1    TAggacGCCG TTTAAGCCtA gTTT
pFh5r2    TAtttgGCCG TTTAAGCCcA gTTT
pFh76r1   TAggacGCCG TTTAAGCCcA cTTT
Consensus TA----GCCG TTTAAGCC-A -TTT
```

Figure 3-2. DNA sequences of repeats in clones pFh5 and pFh76. Clones pFh5 and pFh76 were sequenced in both strands using the Prism™ ready reaction dye primer cycle sequencing kits, containing M13 forward and reverse primers (Applied Biosystems, Foster City CA) and an ABI 373a automated DNA sequencer. Nucleotide sequence data have been submitted to the GenBank database and assigned the accession numbers: PFH5R1: U11819; PFH5R2: U11818; PFH76R1: U11817.

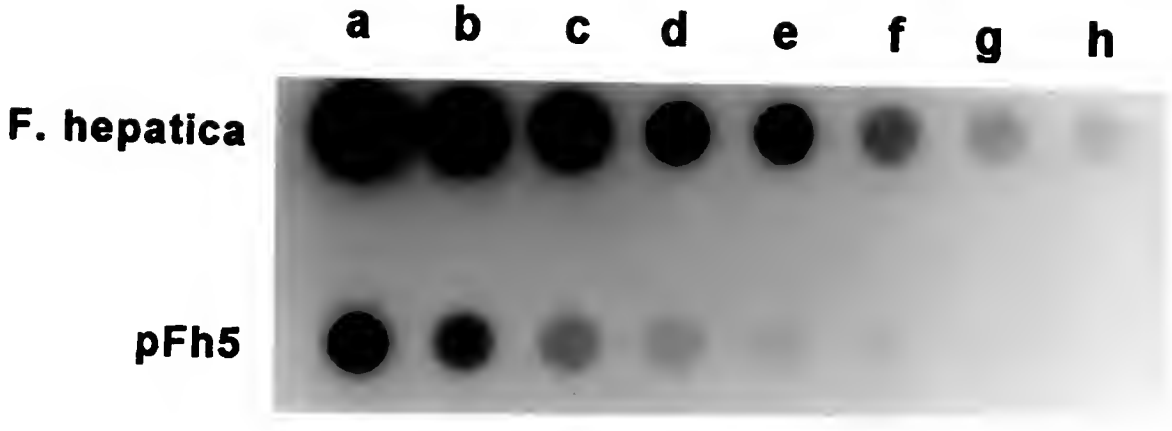

Figure 3-3. Quantitative dot-blot analysis for estimation of the abundance of the 124 bp repeat in the genome of *F. hepatica*. Genomic DNA of *F. hepatica* and PCR amplified insert DNA of pFh5 was blotted onto nylon membrane in doubling dilutions from (a) 100 ng to (h) 0.78 ng for *F. hepatica* and from (a) 1.0 ng to (h) 7.8 pg for pFh5 and hybridized with ^{32}P-labeled pFh5 insert. Intensity of the hybridization signal was measured by storage phosphor imaging (PhosphorImager® 400-S, Molecular Dynamics, Sunnyvale, CA) and the image was quantitated using image analysis software (ImageQuant® Version 3.22, Molecular Dynamics). Hybridization and washing conditions were as in Figure 3-4. under materials and methods. Autoradiographic exposure was for 5 h.

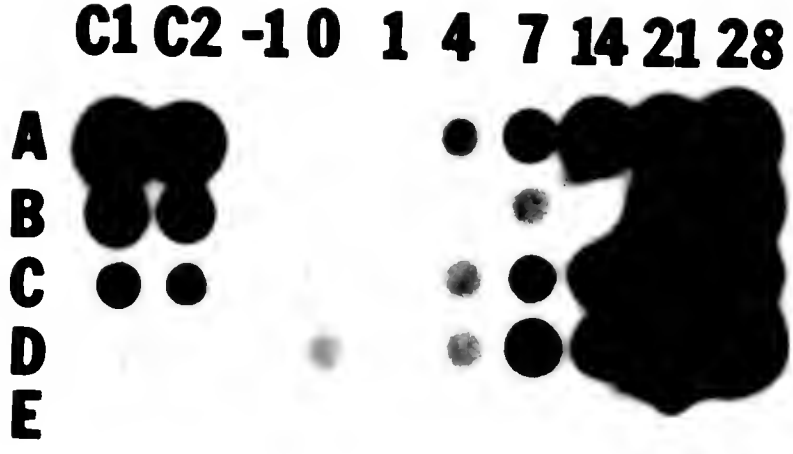

Figure 3-4. Detection of snails infected with *F. hepatica* using probe pFh5. DNA of 4 uninfected snails (-1) and 4 infected snails on days 0, 1, 4, 7, 14, 21 and 28 p.i. (rows A-D). To enable quantitation of hybridization signal of infected snails and calculation of DNA recovery per cent during extraction, genomic DNA of *F. hepatica* in 10X dilutions from 1.0 μg to 0.1 ng was blotted directly (lane C1, rows A-E) or added to individual uninfected snails (lane C2, rows A-E). DNA of all snails was extracted, blotted onto nylon membrane, and hybridized overnight with α^{32}P-dATP labeled probe. Autoradiographic exposure was for 12 h.

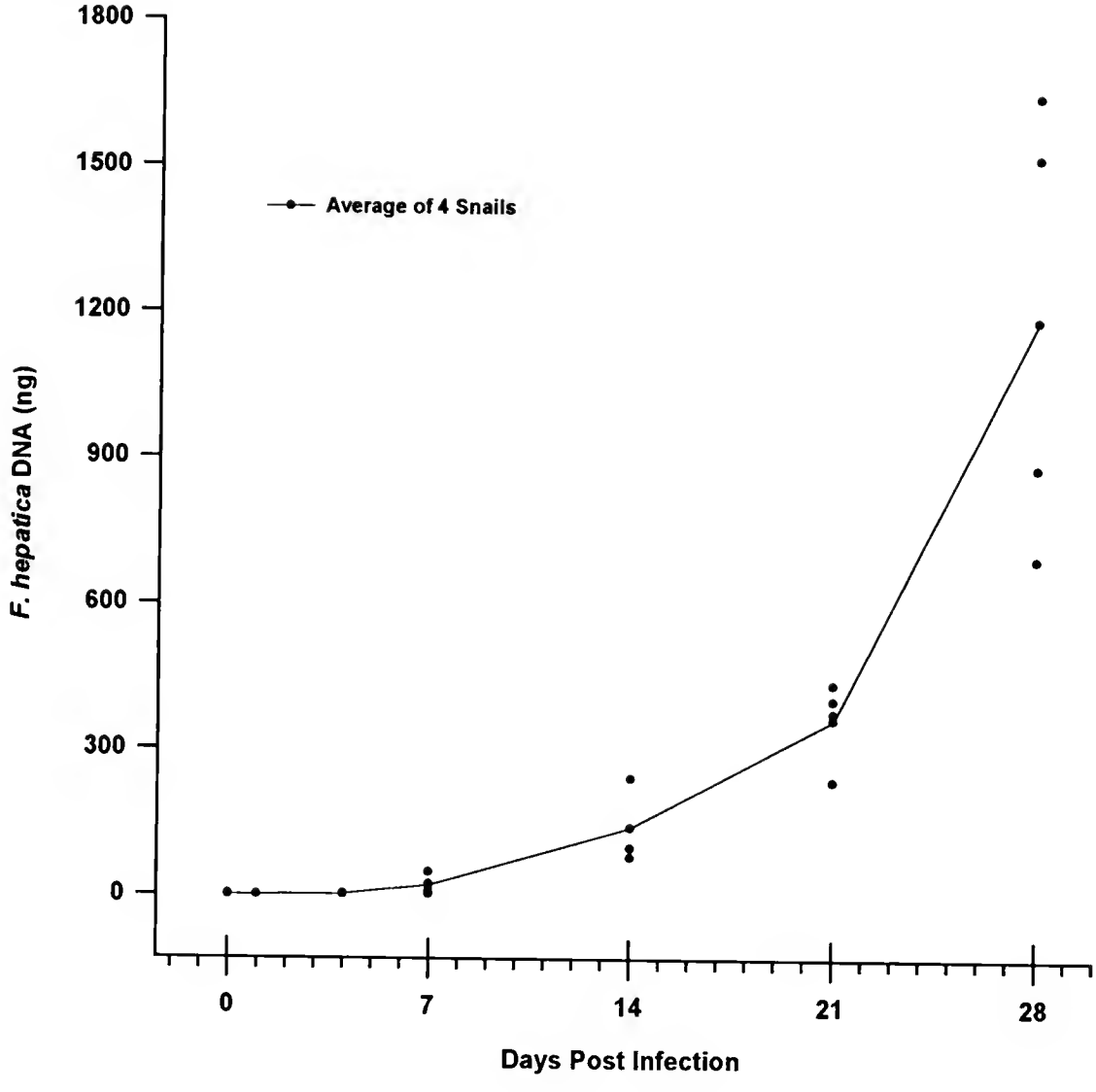

Figure 3-5. Graphical representation of data from Table 3-1. Increase in the total genomic DNA of intramolluscan stages of *F. hepatica* over the first 28 days of infection. Line is through a point representing the average of the 4 individual data points (4 snails).

TABLE 3-1. *F. hepatica* DNA recoveries (ng) from infected snails as determined by phosphor image analysis of blot shown in Figure 3-4*.

	Days Post Infection						
	0	1	4	7	14	21	28
Snail 1	0.20	0.55	2.76	23.18	241.22	376.0	881.56
Snail 2	0.77	0.87	1.31	3.30	ND	401.91	1645.03
Snail 3	0.74	0.23	2.30	9.26	97.89	234.01	692.10
Snail 4	1.71	0.93	2.47	47.86	79.28	434.34	1519.44
Average	0.85	0.65	2.21	20.90	139.46	361.56	1184.53

ND = Not Determined

* Storage phosphor imaging was performed by exposing the hybridized membrane to an imaging screen (Molecular Dynamics, Sunnyvale, CA) for 24 hr. at room temperature. The screen was then scanned using the Molecular Dynamics PhosphorImager® 400-S and the image was quantitated using image analysis software (ImageQuant® Version 3.22, Molecular Dynamics). Background counts were subtracted from all samples and linear regression was performed using standard DNA concentrations vs. observed counts. Results of linear regression were used to calculate actual amounts of *F. hepatica* DNA recovered from each infected snail. True amount of *F. hepatica* DNA would be approximately 67-100% greater than the values reported here since extraction recoveries average 50-60%.

CHAPTER 4
DETERMINATION OF SNAIL INTERMEDIATE HOST INFECTION PREVALENCE WITH *FASCIOLA HEPATICA* USING A DNA PROBE ASSAY

Introduction

Fasciola hepatica, the common liver fluke, is enzootic throughout most of the major livestock producing areas of the world. Control programs aimed at minimizing economic losses from *F. hepatica* are usually based on the properly timed treatment of infected livestock with flukicidal drugs (Armour, 1975; Malone et al., 1982a; Shearer et al., 1986). Because of regional and local differences in seasonal transmission dynamics, the proper timing of these treatments differs for each geographic area. Therefore, before appropriate treatment strategies for the control of liver flukes can be developed for a particular region, the seasonal transmission dynamics must be first determined.

Studies designed to determine seasonal transmission dynamics of *F. hepatica* typically use worm-free sentinel animals, "tracers", that are placed on pasture at 4 or 6 week intervals and necropsied after 6-8 weeks of confinement (Armour, Urquhart, Jennings, & Reid, 1970; Boyce & Courtney, 1990; Craig & Bell, 1978; Hoover et al., 1984). Although tracer studies are often regarded as the most accurate means

for determining seasonal transmission profiles (Armour et al., 1970) they are also extremely expensive to conduct. For example, at the University of Florida, a 2-year single site tracer study with 6 calves placed on pasture at 6 week intervals would cost approximately $75,000. The high cost associated with tracer studies frequently limits them to 1 or 2 years at 1 or 2 sites and can therefore lead to studies where data is incomplete and/or subject to geographic and yearly climatic biases. The great expense of tracer studies also limits the number of investigations that are conducted. This has caused a dearth of knowledge regarding *F. hepatica* transmission dynamics in many areas of the United States and around the world. This is especially important in developing countries where a lack of information regarding parasite transmission prevents the design of rational cost effective control programs.

Tracer studies also frequently include the determination of snail intermediate host bionomics and snail infection prevalences (Boray et al., 1969; Khallaayoune et al., 1991; Malone et al. 1984/85; Ross, 1977). This data is helpful in interpreting the worm acquisition data and enables the researcher to better explain the seasonal transmission patterns that are seen. Unfortunately, existing microscopic techniques lack the sensitivity, specificity and efficiency necessary to obtain accurate and meaningful infection prevalence data in most studies. As a result, the effort to

determine snail infection prevalence is often wasted because the numbers of infected snails detected are too low to have any epidemiologic value (Khallaayoune et al., 1991; Lindsay, 1976; Malone et al. 1984/85; Wilson & Samson, 1971). However, accurate snail infection prevalence data has the potential to be extremely useful in determining seasonal transmission dynamics of trematodes (Christie & Upatham, 1977; Sturrock, 1973). Patterns of *F. hepatica* transmission are the direct result of interactions between the snail intermediate host, the parasite and the environment (Ollerenshaw, 1959). Therefore, data on the bionomics and infection prevalences of these snails can be used to predict changes in seasonal pasture contamination levels and the ensuing risk to grazing livestock. Thus, it is possible to determine seasonal transmission dynamics without using expensive tracer animals, particularly if a method for rapidly testing large numbers of snails for infection is available.

We recently reported the development of a highly sensitive and specific DNA probe for the detection of *F. hepatica* infected snails together with an efficient DNA extraction protocol suitable for large scale testing of field-collected snails (Kaplan et al., 1995). Here we report a modification of that protocol that improves the assay efficiency and makes use of chemiluminescent detection. Using this DNA probe assay we monitored the infection prevalence of more than 5,000 snails on 6 cattle ranches in

Florida during the second year of a 2-year study on snail intermediate host bionomics. Additionally, the infection prevalence of snails on 1 ranch was determined for the first year using the same DNA probe but a different assay system. This report represents the first published use of a nucleic acid probe in the study of *F. hepatica* transmission dynamics and demonstrates the usefulness of this alternate approach.

Materials and Methods

Snail collection and storage (field-collected snails). *Fossaria cubensis* were collected at 3 week intervals from 12 May 1993 to 26 April 1994 using the methods described in chapter 5. Snails were collected from pastures of 6 cattle ranches in Florida: H. E. Wolfe Ranch, St. Johns County; Deseret Ranch, Brevard County; Creek Ranch, Polk County; Rio Ranch, Okeechobee County; John Williams Ranch, Okeechobee County; and Brighton Seminole Indian Reservation (Glades County). At the time of collection, snails were placed in small (~50 ml) plastic jars filled with water obtained at the snail collection site. Jars with snails were then stored on ice in a chest-cooler until brought back to the laboratory. Once at the laboratory, snails were poured into a strainer covered with 2 layers of cheese cloth and rinsed well with tap water to remove as much mud and debris as possible. Snails were then placed back into clean containers with dechlorinated tap water and stored in the refrigerator at 4°C for 1 to 7 days.

Snail processing prior to DNA extraction. Snails were removed from the refrigerator, rinsed again with tap water through cheese cloth and were then placed in a 100 x 15 mm petri dish containing distilled water. Snails were individually removed from the petri dish, measured to the nearest millimeter (rounding upward), and placed on a 15 x 20 cm piece of clean lab-bench protector paper. All snails (or 184 snails, whichever was less) from an individual ranch were measured and counted except for two collections (20 Jan 1994, 06 April 1994) when greater than 184 snails were assayed from the Brighton Seminole Indian Reservation. Microcentrifuge tubes were labelled according to snail size (1 mm to 10 mm) and 200 μl of lysis buffer (8% triton-X 100, 250 mM sucrose, 50 mM TE pH 7.6) for snails $\leq$ 7 mm or 300 μl for snails $\geq$ 8 mm was added. Individual snails were then placed in tubes and were either immediately crushed using plastic pestles or were stored in the refrigerator for 1 to 4 days prior to being crushed. Snails from a single ranch were processed as a unit and were kept at room temperature while being crushed. Crushed snails were then stored in the refrigerator at 4°C if DNA extraction was planned for the same week, otherwise crushed snails were stored in the freezer at either -20°C or -70°C depending upon available storage space.

DNA extraction procedure. Twenty microliters of freshly prepared Proteinase K (10 mg ml^{-1}) was added to crushed snails using a motorized microliter pipet (EDP-Plus®, Rainin

Instrument Co. Inc, Woburn, MA) and samples were incubated in a waterbath at 68°C for 2 to 3 h. For snails ≥ 8 mm, 40 µl of Proteinase K was added and incubations were done for 3 to 4 h. After approximately 1 h of incubation, samples were mixed by gently inverting the tube racks several times. Following incubation, samples were centrifuged for 30 sec at 12,000 x g to pellet sample debris (shell plus undigested snail tissue) and to draw the lysate down and off the tube-lid to prevent sample cross-contamination when the tubes were opened. Lysates of snails ≥ 4 mm were transferred to clean tubes, however, the amount of debris in snails ≤ 3 mm was minimal and these lysates were not transferred. Nucleic acids in the lysates were precipitated by the addition of 800 µl of warm 2% hexadecyltrimethyl ammonium bromide solution (CTAB). Tubes were inverted 10 times to mix, the nucleic acid precipitate was pelleted by centrifugation for 30 sec at 12,000 x g, and the essentially nucleic acid-free supernatant was aspirated using a glass pasteur pipet attached to a vacuum apparatus. 200 µl of 2.5 M NaCl containing 10 mM EDTA was added to dissolve the DNA-CTAB pellets and the samples were placed back into a 68°C waterbath over night to facilitate dissolution. The next morning, racks with tubes were shaken for 10 sec and tubes were centrifuged for 5 sec to draw sample droplets off the lid. A single organic extraction was performed to separate the CTAB from the DNA using 500 µl of chloroform/isoamyl alcohol (24:1) which was stained pink by

the addition of Sudan III (.025 % w/v) (Sigma Chemical Co., St. Louis MO). One hundred and eighty microliters of the aqueous phase (~90% of the sample) was transferred to a well of a 96-well microplate and microplates containing the DNA samples were stored in the refrigerator at 4°C (1 to 7 days) until they were dot-blotted onto nylon membrane. Each microplate contained ≤ 92 test snails, one positive snail control, one negative snail control and 2 empty wells for hybridization controls. All solutions used in the assay in large volumes (≥ 200 µl; lysis buffer, CTAB, NaCl/EDTA, Chloroform) were dispensed using repeat dispensers (Repipet®, Labindustries, Berkeley, CA).

Preparation of snail controls for DNA extraction procedure. Uninfected laboratory raised *Pseudosuccinea columella* were removed from culture tanks and placed in a 100 x 15 mm petri dish containing distilled water for ~1 h. Snails were dried on clean lab-bench protector paper and added to tubes containing 200 µl of lysis buffer. Ten nanograms of *F. hepatica* genomic DNA was added to positive control snails and both positive and negative control snails were crushed and stored at -70°C until needed. Control snails were thawed as needed for each round of snail DNA extractions and were handled exactly the same as sample snails throughout the extraction process.

DNA dot blots. Microplates containing the snail DNA extracts were removed from the refrigerator. Each microplate

had 2 wells that were left empty for use as a positive and negative control for the blotting, hybridization and detection process. 180 µl of 2.5 M NaCl containing 10 mM EDTA (aqueous solution of snail DNA extracts) was added to each control well and the positive control well also had 10 ng of genomic DNA of *F. hepatica* added. Twenty microliters of 2N NaOH (0.2N final concentration) was then added to each well using a motorized microliter pipet (EDP-Plus®, Rainin Instrument Co. Inc, Woburn, MA) and the microplate was placed in a 37°C incubator for 15 min to 3 h to denature the DNA. 100 µl of each sample (one half of volume) was transferred to a nylon membrane contained within a dot blot manifold (Schleicher & Schuell, Keene, NH) using a multichannel pipettor (12-Pette®, Costar Corporation, Cambridge MA). The remaining 100 µl were stored at -70°C. Hybond™ N+ (Amersham Corporation, Arlington Heights, IL) nylon membrane was used when hybridization was with ^{32}P-labeled probe and positively charged nylon membrane (Boehringer Manheim Corporation, Indianapolis, IA) was used when hybridization was with Digoxigenin-labeled probe. Membranes were air-dryed and DNA was crosslinked to the membrane by UV light (Stratalinker, Stratagene, La Jolla CA). Membranes were stored in plastic zip-lock bags until used for hybridization.

Probe preparation and labelling. Probe was PCR amplified from clone pFh5 in a Perkin Elmer Cetus DNA thermal cycler using a standard reaction mixture (AmpliTaq®, Perkin Elmer

Cetus, Norwalk CT) with vector derived primers (TATAGGGCGAATTGGGT, GGTGGCGGCCGCTCTAG). The PCR product was purified by performing a single phenol/chloroform (chloroform/isoamyl alcohol (24:1)) extraction followed by a single chloroform extraction and ethanol precipitation. Recovered probe DNA was resuspended in 100 μl of TE buffer and DNA concentration was estimated by band intensity on agarose gel electrophoresis. Probe was labelled by random primer labelling using either α^{32}P-dATP (111 TBq/mMol, E. I.DuPont De Nemours & Co. Inc., Wilmington, DE; Prime-It® II, Stratagene, La Jolla, CA) or Digoxigenin-11-dUTP (Dig-dUTP; Genius™ 1 DNA Labeling and Detection Kit, Boehringer Manheim Corporation, Indianapolis IA). ^{32}P-labelled probe was freshly prepared for each hybridization, however, Dig-labelled probe was prepared only once. Dig-labelled probe was stored at -20°C and was thawed and used as needed throughout the remainder of the study (8 months).

Hybridization with ^{32}P-labeled probe. Membranes were rinsed in 6X SSC for 5 minutes and partially air dryed. Membranes were then placed in a hybridization cylinder with 20 ml of prehybridization solution (6X sodium chloride, sodium citrate pH 7.5 (SSC), 1.0 mM EDTA, 10X Denhardts, 0.5% SDS, 10 mM $NaPO_4$, 100 μg ml^{-1} denatured salmon sperm DNA) and were incubated for 2 h at 65°C. Labelled probe (12.5 to 25.0 ng) was added to 2.0 mg of salmon sperm DNA and was denatured by placing in a boiling water bath for 5 min. Denatured probe

was added directly to the hybridization cylinder and membranes were hybridized overnight (16 h; ~1.25 ng probe ml^{-1}, ~3 x 10^5 cpm ml^{-1}).

Hybridization with Dig-labelled probe. Membranes (up to 9 at a time) were placed in a covered plastic tray along with 90 ml of prehybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1.0% (w/v) blocking reagent) and incubated for 2 h in a 65°C water bath. Dig-labelled probe was thawed and 450 to 900 ng was removed. Probe was denatured by placing in a boiling water bath for 10 min and was then added to 90 ml of fresh prehybridization solution prewarmed to 65°C (hybridization solution). Prehybridization solution was poured off the membranes and the hybridization solution was added to the container. Membranes were hybridized with probe overnight at 65°C (16 h, 5 to 10 ng probe/ml). Following hybridization, the hybridization solution was collected and stored at -20°C for future use. Each batch of hybridization solution was reused up to 4 times in subsequent hybridizations after denaturing in a 90°C water bath for 30 min prior to each use.

Washing and detection (radioisotopic) of membranes hybridized with ^{32}P-labelled probe. Membranes were washed in a hybridization cylinder at 55°C twice for 15 min in 2X SSC, 0.1% SDS and once in 0.2X SSC, 0.1% SDS for 15 min. Following the last wash, membranes were immediately covered with plastic wrap and placed in a 14 x 17 inch autoradiographic cassette

with autoradiographic film (Hyperfilm™-MP, Amersham Corporation, Arlington Heights, IL) for overnight exposure (~24 h).

Washing and detection (chemiluminescent) of membranes hybridized with Dig-labelled probe. Membranes were washed in a plastic tray once at room temperature for 10 min with gentle shaking in 2X SSC, 0.1% SDS and at 55°C twice for 20 min in 2X SSC, 0.1% SDS and once for 20 min in 0.2X SSC, 0.1% SDS. The last wash solution was poured off and filtered Genius buffer 1 (GB1; 150 mM NaCl, 100 mM Tris-HCl, ph 7.5, 0.3% (v/v) Tween-20) was added to equilibrate the membranes for 1 to 5 min. All steps in the detection process were done at room temperature. Membranes were then transferred to a clean glass dish containing Genius buffer 2 (GB2; 150 mM NaCl, 100 mM Tris-HCl, ph 7.5, 0.3% (v/v) Tween-20, 2% Blocking reagent) and were incubated with gentle shaking for 1 h to block the membranes. GB2 was poured off and anti-Dig-alkaline phosphatase diluted 1:10,000 in GB2 was added to the dish for an additional 30 min incubation with shaking. Following the antibody incubation, membranes were transferred to a clean dish and washed 3 times for 15 min per wash in GB1. After the last wash, GB1 was poured off and Genius buffer 3 (GB3; 100 mM NaCl, 100 mM Tris-HCl, 50 mM $MgCl_2$, pH 9.5) was added to equilibrate the membranes. Lumi-Phos™ 530 (Lumigen Inc., Detroit, MI) was diluted 1:1 with GB3 and added to a clean dish. Membranes were then individually dipped in the

substrate solution until thoroughly wetted. Excess liquid was allowed to drip off and the wet membranes were placed between 2 clear acetate sheets. The top sheet was wiped with a laboratory tissue (Kimwipe®, Kimberly-Clark Corporation, Roswell, GA) to eliminate air bubbles and the covered membranes were placed in a 14 x 17 inch autoradiographic cassette. Autoradiographic film (Hyperfilm™-MP, Amersham Corporation, Arlington Heights, IL) was then placed in the cassette for a 1 1/2 hour exposure or was placed in the cassette the next day for a 15 min exposure.

Scoring of results. Individual samples were scored subjectively on a scale of 0 to 3+ (0 = negative, 1+ = equivocal (very weak positive), 2+ = weak to moderate positive, 3+ = strong positive. Only 2+ and 3+ samples were judged to be true positives whereas 1+ samples were judged to be false positives (see discussion section).

Comparison of the sensitivity of the probe assay to microscopic examination. Laboratory raised *Pseudosuccinea columella* were infected using the methods described in chapter 3. Infected snails were removed at random from a culture tank on days 7 (n = 12), 14 (n = 18), 21 (n = 24), 28 (n = 24) and 42 (n = 18) post-infection. Snails were crushed, quickly examined and then carefully dissected under a binocular dissection microscope (10 to 30 x). Dissections were performed on clean glass microscope slides using sterile 27 G hypodermic needles attached to wooden applicator sticks.

Tissues of all snails in which no larval stages of *F. hepatica* were found were also examined under a compound microscope (40 to 200 x). Following dissection, snail tissue was placed into a labelled microcentrifuge tube containing 200 µl of lysis buffer and stored at -70°C for future testing with the DNA probe assay. The assay was performed exactly as described above for chemiluminescent detection using Dig-labeled probe.

Specificity of the probe assay. Four groups of 72 uninfected laboratory raised *Pseudosuccinea columella* (n = 288) were used in a blinded study to determine the specificity of the probe assay. Eight snails from each group (11%) were selected at random and spiked with genomic DNA of *F. hepatica* as follows: Group 1, 2.0 ng; Group 2, 20 ng; Group 3, 200 ng; Group 4, 2000 ng. Identities of groups and treated samples within groups were unknown to the investigator during the performance of the assay. The DNA probe assay was performed on all snails as described above for chemiluminescent detection using Dig-labeled probe without modification.

Methods used for snails collected during year 1 of the study. Snails collected during the first year of this study were stored in 1N NaOH, 100 mM Tris-HCl pH 8.0 and DNA was extracted using the technique of Hamburger et al. (1987) with minor modifications. Briefly, crushed snails were buffered with 30 µl of 1M Tris-HCl, pH 7.6 and neutralized with 75 µl of 2N HCl. Tubes were placed in a boiling water bath for 5 min and centrifuged at 12,000 g for 30 sec to pellet snail

debris. Supernatant was transferred to a clean tube, DNA (along with other sample components) was precipitated with ethanol and the DNA pellet was dissolved in TE buffer. One half of each sample was then loaded onto a 1.0% agarose gel and the DNA was separated by gel electrophoresis (60V, 30 min). DNA was transferred to nylon membrane by southern blot (Southern, 1975) and membranes were then processed as those for dot blots as described above.

Statistical Methods. Sensitivity of the probe assay vs. dissection and vs. crushing were compared separately for days 7, 14, 21 and 28 post-infection using the McNemar paired X^2 test. The prevalence of infection for snails of different size classes was compared using a X^2 test of independence. Data for groups 1 mm and 2 mm were combined as were data for groups 8 mm, 9 mm, and 10 mm because too many 0 expected values were present if each group was included separately in the analysis. Both analyses were performed on a personal computer using proprietary software (SigmaStat®, Jandel Scientific, Corte Madera, CA). Ninety five percent confidence intervals were calculated for assay sensitivity and specificity and for snail infection prevalence (Miller, 1966).

Results

Sensitivity and specificity of DNA probe assay. Using radioisotopic detection, the probe assay easily detected 1.0 ng of purified *F. hepatica* genomic DNA with overnight

autoradiographic exposure. This level of sensitivity is sufficient to detect a single miracidium; the minimum biological unit of the parasite. With chemiluminescence we improved on this level of sensitivity whereby 1.0 ng of *F. hepatica* DNA was detected with an even stronger signal intensity after only a 90 min exposure. This enhanced the signal to background ratio further increasing the ease with which early infections were detected. To estimate the sensitivity and specificity of the assay when examining large numbers of field-collected snails of unknown infection status, a blinded study was performed using 288 laboratory reared snails, 32 of which had *F. hepatica* DNA added in amounts of 2 ng, 20 ng, 200 ng, or 2000 ng. All 32 of these snails were detected for a sensitivity of 100%. Additionally, all known positive samples (n=219) assayed during the course of the study were detected. The assay therefore has a sensitivity of 100% (95% CI = 97.8 to 100).

In the same blinded study of 288 snails, specificity varied depending upon the quantity of *F. hepatica* DNA present in positive samples. When 200 ng or less DNA was present in positive samples, specificity was 100% (95% CI = 97.5 to 100) with 192 of 192 negative snails correctly identified. However, when 2000 ng of DNA was present, 1 negative sample showed a weak positive signal. Specificity in this group was 98.4 (95% CI = 90.5 to 99.8) with 63 of 64 negative snails correctly identified. Overall then 255 of 256 negative snails

were correctly identified as negative for a specificity of 99.6 (95% CI = 97.4 to 99.9).

Comparison of the DNA probe assay with microscopic examination. Infected snails were crushed and examined microscopically both with and without dissection on days 7, 14, 21, 28 and 42 post-infection. Snails were then placed in tubes and analyzed using the probe assay. On days 7, 14 and 21 (prior to natural snail mortality), 49 of 54 snails (91%) were detected with the probe assay. However, experimentally induced infections are never absolute, and in our laboratory we never recorded greater than 90% infection takes in the numerous experimental snail infections that we have performed. This fact combined with the established 100% sensitivity of the assay led us to conclude that only those snails shown to be positive with the probe were actually infected. This number was then used as a baseline to compare the results obtained by microscopy (Table 4-1, Figure 4-1). The probe assay was significantly more sensitive than crushing alone on days 7, 14, 21 and 28 post-infection and was significantly more sensitive than dissection on days 7 and 14. By day 21 the latter difference was only marginally significant and by day 28 there was no difference.

Prevalence of infection in field-collected snails. The prevalence of infection varied greatly between the 6 ranches and large numbers of infected snails were found on only 2 of the ranches (Table 4-2). This finding is in accord with data

collected on herd prevalence by microscopic examination of feces (see chapter 5). A total of 5,246 snails were assayed for infection and 79 or 1.51% were infected with *F. hepatica*. On the Brighton Seminole Indian Reservation pasture, where ranch management and ecological conditions were the most optimal for fluke transmission of the 6 ranches studied (100% herd prevalence based on examination of 20 samples, on 16 Nov 1993), 49 of 1,589 or 3.1% of snails were infected with *F. hepatica*. Snails collected from the Seminole Indian Reservation pasture during the first year of the study (prior to the development of the assay used to obtain the above results) were processed using a different system. Snails from other ranches during this period were not assayed. Of 1037 snails assayed using this other technique, only 7 were infected (0.68% prevalence). This finding does not agree with data collected on herd prevalence by microscopic examination of feces (64% herd prevalence based on examination of 25 fecal samples, on 18 Dec 1992). The prevalence of snail infections on the Seminole Indian Reservation pasture for the 2 years examined was significantly different ($P = <0.0001$) using the 2 different assays.

The prevalence of infection significantly differed ($p = 0.0001$) between successive size groupings varying from 0% for 1 mm snails to 18.5% for 9 and 10 mm snails (Figure 4-2, Table 4-3). Snails 6 mm or larger accounted for only 21.7 of all snails assayed but represented 57% of all infected snails.

Overall, only 0.8% of snails 1 to 5 mm were infected whereas 3.9% of snails 6 to 10 mm were infected. This shift in infection prevalence is illustrated in Figure 4-3 where the size distribution of the infected population of snails is skewed to the right as compared to the total population of snails.

Discussion

This report describes the first use a nucleic acid-based technique for determining the prevalence of snail intermediate host infections in an epizootiologic study of *F. hepatica*. The DNA probe assay can detect infected snails immediately following miracidial penetration and the probe does not cross hybridize with DNA of *Fascioloides magna*, *Paramphistomum liorchis*, or *Heterobilharzia americana*; trematodes that share the same snail intermediate hosts and have overlapping enzootic ranges with *F. hepatica*. Sensitivity of the probe assay is 100% and specificity is greater than 99%. The assay uses a chemiluminescent detection system that is quicker, safer, more sensitive and less expensive than radioisotopic detection. Chemiluminescence also increases the consistency of the assay; enough probe for an entire study can be labeled in a single reaction, the yield of labeled probe determined and the labeled probe then can be stored for a year. Once this assay was converted to chemiluminescence, the same batch of labelled probe was used for every hybridization for the

remainder of the study. With radioisotopes, probe was freshly labeled for each hybridization (half life of ^{32}P = 14 days). The practical consequences of preparing labeled probe for each hybridization is seen in Figure 4-4. ^{32}P-dATP labelled probe with low specific activity resulted in a blot with poor sensitivity. To avoid this, the specific activity of each batch of radiolabeled probe needs to be determined, further increasing the time and expense of using radioisotopes as well as the potential for dangerous radioactive exposure.

Using chemiluminescent detection, the entire assay was performed in a clinical parasitology laboratory without the need for specialized equipment or facilities that only would be available in a recombinant DNA laboratory. The only equipment needed to complete the assay are: a waterbath; a microcentrifuge; a table-top shaker; a dot blotter with vacuum pump; a few pipettors; and a -20°C freezer and refrigerator for storage of samples and reagents. This equipment is fairly standard, inexpensive and readily available for most laboratories. Additionally, samples were kept at room temperature throughout the entire procedure. This greatly improves the ease of sample handling as compared to assays that require samples to be kept on ice. Samples frequently were left at room temperature for several hours during many of the steps of the assay without any apparent DNA degradation or loss of sensitivity. The assay is also very quick and inexpensive to perform. The entire assay can be completed for

the low cost of about U.S. $0.33 per snail and takes only about 3 min per snail to complete when large numbers of snails (~400) are assayed at a time. Because of the time efficiency and low cost of this assay, over 5,000 snails were examined in only a few months.

Before making claims of superiority for a new diagnostic technique, it is important to directly compare that technique to existing ones. We did this by examining experimentally infected snails both by microscopy and using the DNA probe assay on days 7, 14, 21, 28 and 42 post-infection. Ninety one percent of experimentally infected snails were detected with the probe. Because a 90% infection rate is about the best that can be achieved in a group of experimentally infected snails and because we clearly demonstrated a 100% sensitivity for the probe assay, we concluded that only those snails that were probe-positive were actually infected. Using this value as a baseline we then compared the sensitivity of crushing alone or crushing with dissection. No snails that were probe-negative were found to be infected by microscopy. The probe assay was significantly more sensitive than crushing alone on days 7, 14, 21 and 28 post-infection. The difference in sensitivity was not as great when snails were dissected. The probe assay was significantly more sensitive than dissection on days 7 and 14, but by day 21 the difference was only marginally significant and by day 28 there was no difference. However, only 24 snails were compared on day 21 so it is

likely that if more snails were examined that differences between the two techniques would become significant. It is also worth noting that during dissection the microscopist had *a priori* knowledge that most all snails would be infected. Dissection was performed with extreme thoroughness and all snails not diagnosed as infected under low power microscopy were then examined under a compound microscope. This was a slow and tedious process with individual dissections taking about 6 to 8 min to complete. In examining large numbers of field-collected snails, most of which would be uninfected, this level of completeness would be very impractical and realistically would not be done. Therefore, the sensitivity for dissection obtained in this experiment is an overestimate of what would be seen under realistic conditions. The true sensitivity for dissection is probably intermediate between the values we determined for crushing and dissection. The probe assay is therefore both quicker and significantly more sensitive than dissection. Additionally, the probe assay has 100% specificity against *F. magna* and *Paramphistomum* spp. whereas with dissection, sporocyst and redial stages of these 2 trematodes are not readily distinguished from those of *F. hepatica*.

Under controlled conditions with relatively small numbers of known infected and uninfected snails, sensitivity and specificity of the assay are both 100%. However, when assaying large numbers of snails of unknown infection status,

there is a possibility of introducing laboratory error. Since this assay has far better sensitivity and specificity than any other method, there is no gold standard to compare this assay with. We therefore felt it was important to establish the sensitivity and specificity of the assay when used for its intended purpose e.g. examining large numbers of field-collected snails of unknown infection status. To do this we performed a blinded study using 288 laboratory reared snails, 32 of which had *F. hepatica* DNA added in amounts of 2 ng, 20 ng, 200 ng, or 2000 ng. Results from that experiment demonstrated a 100% sensitivity and a specificity that is not absolute but rather changes with the prevalence of mature snail infections. When 200 ng of *F. hepatica* DNA was added to 11% of samples, an amount of DNA approximately equal to a 14 to 21 day old infection, specificity was 100%. However, when 2000 ng of *F. hepatica* DNA was added, an amount approximately equal to an infection approaching patency, specificity decreased to 98.4 due to a single weak false positive result. Of the 5,246 snails assayed in this study, 25 were rated as equivocal (1+, very weak positive). Twenty of these were in wells adjacent to snails recorded as strong positive (3+) and most probably represented false positives. The other 5 likely are true positives, but for purposes of consistency were assigned a score of equivocal and not reported as positives. However, even assuming that all 25 were false positives, the overall specificity of the assay in this study still would be

99.5% if we accept that all 2+ and 3+ positives were true positives.

One ranch (Rio) had only 1 infected snail (2+, moderate positive signal) out of 1410 assayed (0.07% prevalence). Although this ranch has a history of infection with *F. hepatica*, no eggs were detected when cattle feces were examined. It is unlikely that liver flukes were eradicated from this ranch so it is probable that this one infected snail was a true positive. However, if we take a conservative view and assume that this was a false positive, specificity on this ranch would still be greater than 99.9%. Therefore, when infection prevalence is low, specificity approaches 100%. When infection prevalence is high, specificity is slightly lower due to occasional sample cross contamination. From a practical standpoint, however, a small percentage of false positives under high prevalence conditions has little epidemiological importance because: (1) the predictive value of a positive test increases as the prevalence of disease increases (Courtney, 1990); and (2) infection prevalence is a population measure, test results of individual snails are immaterial.

Using this DNA probe assay we found a much higher infection prevalence in snails than did other investigators who used microscopic techniques. Wilson and Samson (1971) found only 1 infected snail out of approximately 1,000 examined over a four year period (0.1% prevalence). During

this period, sheep grazing the pastures where snails were collected averaged 49% prevalence and cattle averaged 30% prevalence. Malone et al., (1984/85) found only 2 infected snails of a total of 1500 collected over a 3 year period (0.13% prevalence) on a pasture where infection prevalence in cattle reached 96% (13.2 EPG). Khallaayoune et al., (1991) also found only 2 infected snails over a 3-year period out of 1188 snails examined (0.17% prevalence) in a study where 10% of tracer lambs died of clinical fascioliasis. An exception to this is in some studies done on pastures grazed by sheep where high infection prevalences were sometimes found (Boray et al., 1969; Ollerenshaw, 1959; Smith, 1981). However, the transmission potential of *F. hepatica* by sheep is much greater than that by cattle. It is estimated that egg output by sheep in a given area is approximately 140 to 200 times greater than that by cattle (Boray et al., 1969), thus the likelihood of a snail becoming infected is much greater on pastures grazed by sheep.

In this study, the prevalence of infection increased for each successive size class reaching 18.5% for 9 and 10 mm snails. Overall, snails 6 mm or larger accounted for 57% of all infected snails but only represented 21.7% of all snails assayed. This pattern of infection is similar to that reported by Ollerenshaw (1959) and Smith (1984) in studies where thousands of snails collected from pastures grazed by fluke-infected sheep were dissected under microscopy. In both

studies, infection prevalence, redial burdens and the proportion of rediae containing mature cercariae all increased with increased shell length. This pattern with large snails having significantly greater levels of infection than small snails can be explained 3 ways. First, for a given population of snails, size is directly proportional to age. Larger snails will have been on pasture longer than small snails and would therefore have had more opportunities to become infected. Second, large snails are more likely to be penetrated by miracidia due to increased chemoatractiveness. Third, infected snails may grow larger than their uninfected counterparts due to a parasite induced partitioning of nutritional resources away from reproduction and toward growth (Wilson & Denison, 1980). The observed trend toward greater infection prevalence in larger snails has 2 major practical implications: (1) the number of snails that need to be assayed in a study of snail bionomics and infection prevalence can be greatly reduced by selecting proportionately more large snails for analysis; (2) small snails can be assayed in groups without modifying the assay and without compromising the quality of the data obtained. Although group-testing of snails was not done in our study, it should be easily accomplished since the biomass of a snail is an exponential function of its length (Smith, 1984).[1] Therefore, 18 3 mm

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[1]Snail biomass estimated by $y = 0.024x^{2.656}$: where y = dry weight biomass of soft parts (mg), and x = shell length (mm).

snails are actually smaller in terms of total snail biomass than a single 9 mm snail.

Recently, Rognlie, Dimke, & Knapp (1994) reported an assay for the detection of *F. hepatica* infected snails using reverse transcriptase-polymerase chain reaction (RT-PCR). This assay although extremely sensitive has several problems that limit its usefulness as a diagnostic assay. First, it cross-hybridizes with *F. magna*. This parasite is prevalent throughout the entire enzootic range of *F. hepatica* in the U.S. and *F. magna* will readily infect the same *Fossaria* (Lymnaea) species that serve as intermediate hosts for *F. hepatica* (Foreyt & Todd, 1978; Krull, 1934; Malek, 1980; Olsen, 1949). In the Gulf Coast region of Texas, *F. bulimoides* is the primary intermediate host for both trematodes (Foreyt & Todd, 1976b). Additionally, in areas with large populations of white-tailed deer, the infection prevalence of *F. magna* in cattle may even exceed that of *F. hepatica* (Craig & Bell, 1978). Second, the assay was never tested with DNA of *Paramphistomum (Cotylophoron, Calicophoron)* spp., another trematode that shares the same intermediate hosts and same enzootic range with *F. hepatica* in the Americas (Castro-Trejo et al., 1990; Malek, 1980; Malone, Fehler, Loyacano, & Zukowski, 1992; Swanson et al., 1952). On the ranches studied in our project, the prevalence of infection with *Paramphistomum spp.* in cattle was consistently greater than the infection prevalence with *F. hepatica*; the average

egg shedding index for *Paramphistomum spp.* was 5.9 times that of *F. hepatica*.[2] This relative prevalence has also been reported in Louisiana cattle (Malone et al., 1992; Zukowski, Wilderson, & Malone, 1993). Therefore, without specificity or knowledge of specificity against either of these 2 trematodes, a nucleic acid probe assay can not be relied upon to give credible data on *F. hepatica* infection prevalence in snail intermediate hosts.

The RT-PCR assay is also very arduous and expensive to perform. Total RNA is first extracted and purified from snails using a laborious protocol, cDNA is prepared and then PCR is performed using rRNA derived primers. PCR products are visualized by agarose gel electrophoresis, DNA is transferred to nylon membrane, and results are finally obtained by hybridization with radiolabeled probe. This lengthy assay precludes the efficient analysis of very large numbers (thousands) of snails that are needed to obtain statistically meaningful data on snail infection prevalence. Additionally, although the authors do not specify the cost of their assay per snail, the expense of materials used in this assay are significant. Finally, their assay was never tested for sensitivity or specificity using large numbers of snails of unknown infection status. PCR-based assays because of their excellent sensitivity are also prone to specificity problems

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[2]Egg shedding index = herd mean EPG x herd prevalence (Malone et al., 1992)

due to amplicon contamination of reagents and/or sample to sample cross-contamination. To reduce the chances of contamination, samples need to be handled with meticulous vigilance further decreasing sample handling efficiency and increasing the time required to complete the assay.

The study on snail intermediate host bionomics was started prior to having a probe for the detection of infected snails and an assay to use the probe. During the first year of the study, snails were stored in 1N NaOH based on a published report (Hamburger et al., 1987) and preliminary experimental results indicating that the technique would work satisfactorily in a dot blot assay. Later experiments showed that *F. hepatica* DNA could not be detected in snails using this technique and an alternative technique was developed. However, an effort was made to salvage the samples stored in 1N NaOH by precipitating the DNA and then purifying the DNA using gel electrophoresis. *F. hepatica* DNA was then detected by southern hybridization since the portion of the sample which prevented detection by dot blot hybridization was now separated from the DNA. Small numbers of control samples gave good results after optimizing this system, however, when actual samples were processed in large numbers, results were much less consistent. Additionally, no proper controls existed i.e. known infected snails stored similarly, therefore confidence in the results is not great. Additionally, DNA degradation was apparent following agarose gel

electrophoresis, but whether this is important is debatable since the fragment of DNA detected by the probe is only 124 bp in length. Therefore, the differences seen between the 2 years on the Seminole Indian Reservation pasture could either be due to a real difference that existed between the 2 years or a difference in sensitivity between the 2 assay systems. Because of this uncertainty, it is best not to over-interpret the results. Clearly, however, more years of data using the new and proven assay are desirable.

With tracer studies, data is very expensive to obtain and is subject to geographic and climatic bias if limited to 1 or 2 sites for 1 or 2 years. On the other hand, data from studies in which snail intermediate host bionomics and infection prevalences are determined from multiple sites for multiple years is much less expensive to obtain and is much less likely to be effected by such biases. Under circumstances of unlimited financial resources it would be ideal to combine all 3 types of data: fluke acquisition by tracer animals; snail intermediate host bionomics; and snail intermediate host infection prevalences, for several years at several sites. However, research dollars for these types of studies are becoming increasingly difficult if not impossible to obtain. The cost of a tracer study done for 3 years at 3 separate locations would be greater than $300,000. Using the alternative approach suggested here without the use of tracers, the cost of a 3 year study on 3 sites would be less

than $15,000. This cost is easily within the resources of most laboratories and could be funded from small grants available through both governmental and private sources. The low cost and procedural ease of this DNA probe assay should enable veterinary parasitologists to initiate studies on *F. hepatica* transmission dynamics wherever a need exists. This assay can be easily performed in modestly equipped facilities including field research stations and only minimal experience in molecular techniques is required. With improved knowledge of *F. hepatica* transmission dynamics, strategic control programs can be developed that maximize both the animal health and economic benefits of treatment.

Table 4-1. Comparison of the DNA probe assay with microscopy.

Days PI	No. Examined	Probe +	Dissection +[†]	Crushing +
7	12	11[a]	0[b]	0[b]
14	18	16[a]	3[b]	0[b]
21	24	22[a]	17[•]	3[b]
28	24	20[a]	19	13[b]
42	18	12	12	12

[†] statistical differences between dissection and crushing were not determined
[ab] different superscripts represent statistically significant differences ($p < 0.05$)
[•] difference from [a] is marginally significant ($p = 0.0736$)

Table 4-2. Snail intermediate host infection prevalence for six Florida ranches.

Ranch	No. Assayed	No. Infected	% Infected (Prevalence)
Creek	470	2	0.43
Deseret	784	6	0.77
Rio	1410	1	0.07
Seminole	1589	49	3.08
Williams	747	20	2.68
Wolfe	246	1	0.41
Total	5246	79	1.51

Table 4-3. Combined snail infection prevalence data by size class from six Florida cattle ranches.

Snail size (mm)	No. Assayed	No. Infected	% Infected	% of Total Infected	% of Total Assayed
1	87	0	0.0	0.0	1.7
2	627	2	0.3	2.5	12.0
3	979	8	0.8	10.1	18.7
4	1293	12	0.9	15.2	24.6
5	1119	12	1.1	15.2	21.3
6	678	17	2.5	21.5	12.9
7	330	14	4.2	17.7	6.3
8	106	9	8.5	11.4	2.0
9	20	4	20.0	5.1	0.4
10	7	1	14.3	1.3	0.1
Total	5246	79	1.5	100	100
1-5	4105	34	0.8	43	78.3
6-10	1141	45	3.9	57	21.7

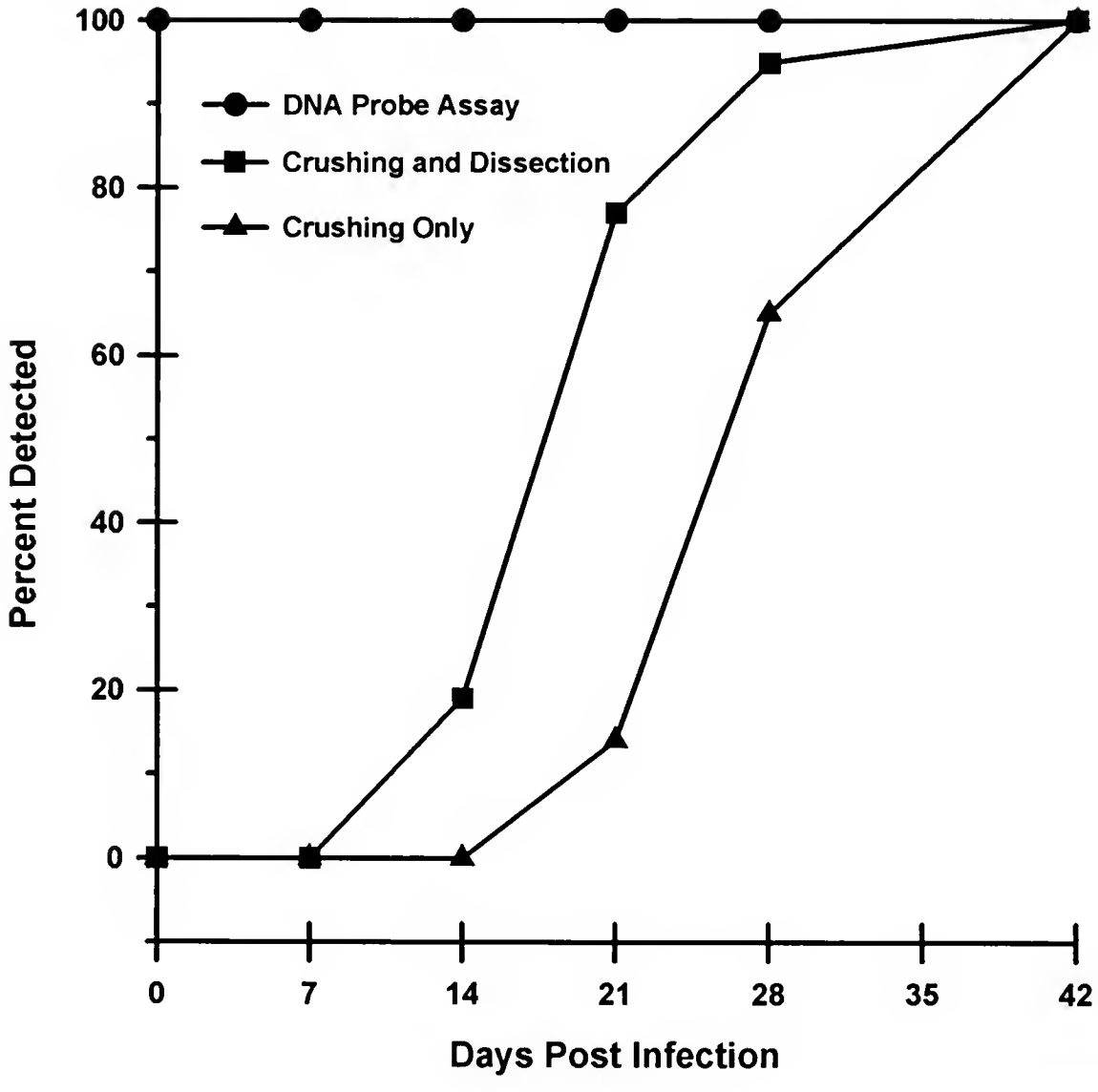

Figure 4-1. Comparison of the sensitivity of the DNA probe assay with crushing alone and crushing with dissection.

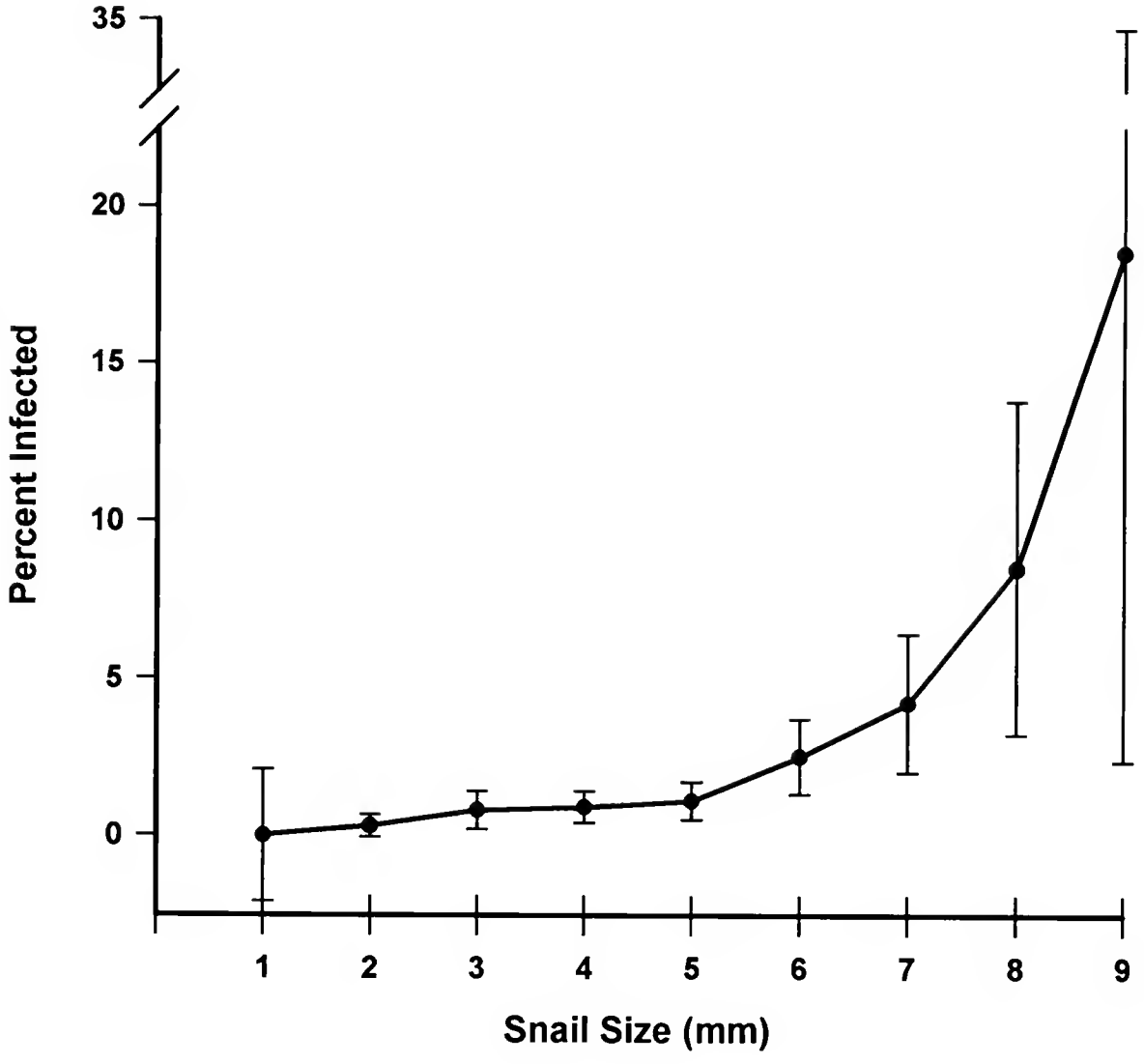

Figure 4-2. Infection prevalence of *Fossaria cubensis* by size class.

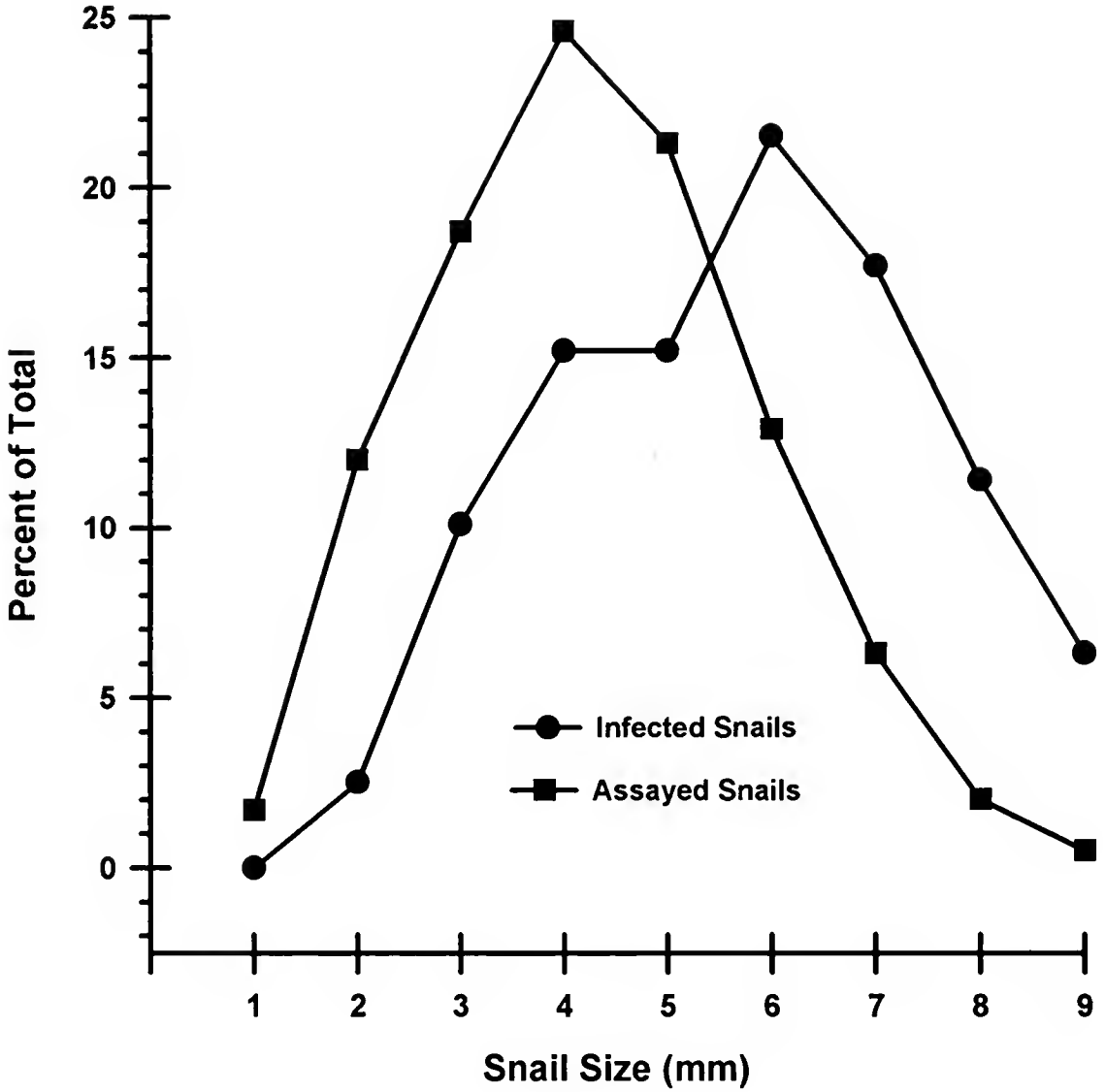

Figure 4-3. Size distributions of the total and infected snail populations.

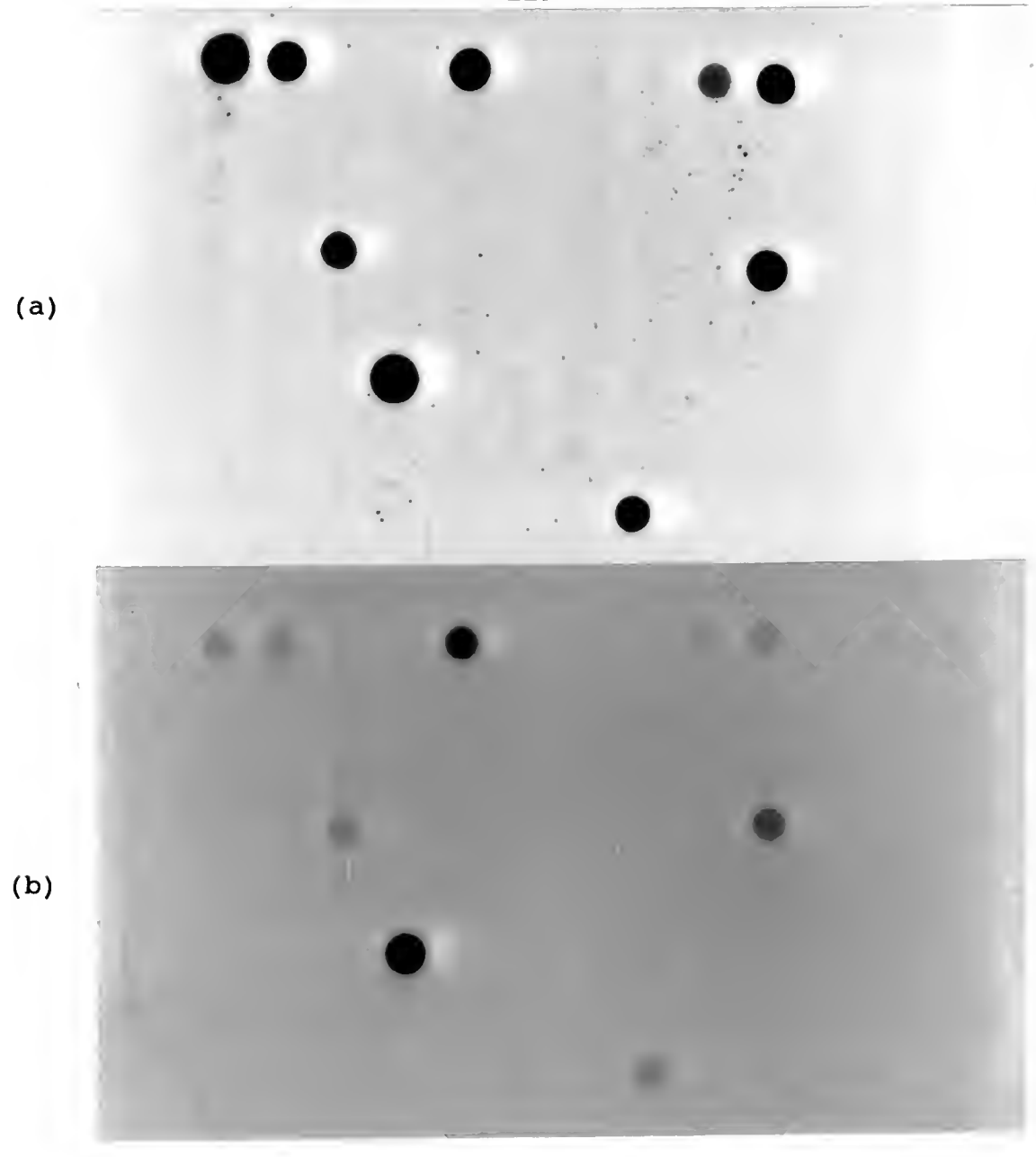

Figure 4-4. Autoradiagraphs of snail dot blots. Duplicate samples (snail DNA extracts) were applied to nylon membrane as described in materials and methods. (a) Blot was hybridized with probe pFh5, labelled using Digoxigenin-dUTP. (b) Blot was hybridized with probe pFh5, labelled using ^{32}P-dATP. The 2 dots in the left-most corner of the top row of the membrane are positive control wells (5.0 ng genomic DNA of *F. hepatica*) and the 2 dots to the right of these are negative control wells. Seven of the remaining 92 wells contained DNA extracts of infected snails as indicated by the positive hybridization signal.

CHAPTER 5
DYNAMICS OF *FOSSARIA CUBENSIS* POPULATIONS IN FLORIDA AND THE RELATION TO THE EPIDEMIOLOGY AND CONTROL OF BOVINE FASCIOLIASIS

Introduction

Bovine fascioliasis is enzootic throughout most of peninsular Florida (south of the Suwannee River) where it is one of the most important health problems of cattle (Shearer et al., 1986). Over 90% of Florida's 1.1 million beef brood cows are pastured in this region (Florida Department of Agriculture and Consumer Services, 1994), and based upon liver condemnations at slaughter, approximately 68% of these cows are infected with liver flukes. It is estimated that proper fluke control applied to these one million plus beef cows has the potential to increase net income to the cattle industry in Florida by approximately 11 to 22 million dollars annually (Simpson & Courtney, 1990).

Before cost effective control strategies for liver flukes are developed, the seasonal transmission dynamics first must be determined. Early studies on the epidemiology of *F. hepatica* in Florida primarily addressed the parasite biology and the ecology of the snail intermediate hosts (Batte, Swanson, & Murphy, 1951; Batte & Swanson, 1951). No data was reported on the seasonal population dynamics of the snail

intermediate hosts or on the patterns of seasonal transmission to the ruminant hosts. Recommendations for the control of fascioliasis were based on proper management practices including a program of snail eradication and treatment of infected cattle with hexachlorethane (Batte & Swanson, 1951; Batte et al., 1951; Swanson et al., 1952).

Boyce & Courtney (1990), determined the seasonal transmission dynamics of *F. hepatica* in north central Florida using tracer sheep. Fluke transmission occurred from December to June with February, March and April as the peak months. This seasonal pattern was repeated with only minor variation over 3 years. No summer transmission occurred in any of the 3 years: in 1986 and 1987 transmission ceased in May and in 1984 and 1985 transmission ceased in June. Fluke transmission resumed during the late autumn or early winter: November (1986), December (1987), or January (1985). This pattern of transmission is very similar to that reported for Louisiana and Texas (Craig & Bell, 1978; Malone et al., 1984/85), the other states of the Gulf coast where *F. hepatica* is highly enzootic.

Recommendations for the treatment and control of liver flukes for all Florida cattle were made based upon this seasonal transmission pattern for north central Florida (e.g. absence of summer and early autumn transmission), (Courtney et al., 1985, Shearer et al., 1986). It was suggested that cattle on ranches where *F. hepatica* is endemic be treated with

a flukicide once annually in the early autumn with an optional spring treatment based on local parameters of risk, e.g. amount of yearly rainfall and history of fluke infection on individual properties. However, the climates of northern and southern Florida differ substantially: northern Florida has a warm temperate climate; whereas southern Florida, where 66% of Florida's beef cows are pastured, has a subtropical climate.

Recently, a climate forecast model was developed for prediction of relative fluke risk in different years, and thus whether there is a need to treat cattle once or twice annually (Malone, 1989; Malone et al., 1987). This forecast model also predicts the seasonality of fluke transmission for a given region. Although not verified for Florida, this forecast model correctly predicted in a retrospective study (Malone & Courtney, unpublished data) the transmission profile (i.e. the seasonality and intensity of fluke transmission) that had been reported by Boyce and Courtney (1990) in north central Florida. However, this same model predicts that there may be two transmission seasons (winter and summer) in subtropical southern Florida (Malone & Craig, 1990).

If fluke transmission in subtropical southern Florida occurs mostly in the winter and summer then treatment of cattle in early autumn and spring would be ill advised. Therefore, it is of extreme importance to the cattle industry of southern Florida to determine the seasonal transmission dynamics of *F. hepatica* in this region. Additionally, the

seasonal transmission dynamics should be studied in central Florida to ascertain any differences that may exist in this transitional area. With these goals in mind, a 2-year study was designed to determine the seasonal transmission dynamics of *F. hepatica* for the different climatic regions of Florida.

To minimize the chances of geographic bias in the data, 2 ranches each from northern, central and southern Florida were selected for study. In addition, a third ranch with a continuously flowing artesian well was included for southern Florida. On some cattle ranches, water sources are augmented by continuously flowing artesian wells during periods of dry weather. It is known from earlier studies that runoff from artesian wells may modify snail habitat to the extent that snails may remain active during periods of drought (Batte & Swanson, 1951). Because the presence of continuously flowing wells could potentially alter seasonal transmission patterns, this additional ranch was also studied.

The seasonal transmission dynamics of fascioliasis is often established for a particular area through the monthly slaughter of sentinel animals ("tracers") grazing known fluke-infested pastures, but this is an expensive process (see chapter 4). Using tracer animals in this 2-year study would have cost greater than $500,000. This level of funding would be difficult if not impossible to procure. Therefore, an alternative approach was required. Since transmission of *F. hepatica* closely follows the availability of its snail

intermediate hosts, it is also possible to determine seasonal transmission of *F. hepatica* indirectly by studying the population dynamics and infection prevalence of these snails. In Florida, the lymnaeid snails *Pseudosuccinea columella* Say and *Fossaria cubensis* Pfeiffer serve as the snail intermediate hosts of *F. hepatica* (Swanson et al., 1952) with *F. cubensis* being the more prevalent and the intermediate host of primary importance. The latter approach, i.e. the determination of the bionomics of *F. cubensis* populations, was taken in this project because it has the advantage of allowing the investigation of more sites at a far lower cost than can be done using sentinel animals (see chapter 4).

Material and Methods

Snail habitat data. Prior to snail collections, a data sheet was completed for each snail habitat. Included were: site ID; date; collection number; time; habitat description; weather description; soil moisture level of habitat - dry, moist, wet or saturated/submerged; air temperature; water temperature; soil temperature at the surface, 3.5 and 10 cm depths; other snail species present; whether cows were on pasture; and notes/comments. Soil surface moisture was categorized using a visual, ordinal scale (Smith & Wilson, 1980) based on the following criteria. (1) Saturated/submerged: soil surface was partially or completely covered by a layer of standing water. When soil was only

partially covered with water, areas of exposed mud were fully saturated i.e. when a finger depression was made in the mud it rapidly filled with water. (2) Wet: soil surface was dark in color with an obvious film of water present. Depressions in soil were frequently filled with water. (3) Moist: soil surface was dark in color but with no surface film of water. Soil was damp to the touch. (4) Dry: soil was pale in color and dry to the touch.

Snail collections. Snails were collected from pastures of 7 cattle ranches in Florida: (a) northern Florida, H. E. Wolfe Ranch, St. Johns County and University of Florida Dairy Research Unit, Alachua County; (b) central Florida, Deseret Ranch, Brevard County and Creek Ranch, Polk County; (c) southern Florida: John Williams Ranch, Okeechobee County, Brighton Seminole Indian Reservation (Glades County), and Rio Ranch, Okeechobee County (artesian well). Each study site was first visited for the purpose of data collection in April or May 1992 with the exception of Creek Ranch which was not added to the study until October 1992. Snail populations were monitored every three weeks for 2 years at each study site using timed and quadrant collection methods (Malone et al., 1984/85; Smith, 1981). Timed collections consisted of a total count of snails observed during a 7.5 minute visual search of each habitat. When snail populations were so large that the speed with which snails were collected was the only factor limiting the number collected, a 2.5 minute search was done.

This number was then multiplied by 3 to standardize counts. Two hundred and fifty snails was about the most that could be collected in 7.5 min (1 snail per 1.8 sec) and this value was used as a maximum cut off for a single timed search. Quadrant collections consisted of collecting all snails found on soil or vegetation within a 0.1 m^2 (12.5 in. x 12.5 in.) area of the habitat. Timed collection data gives a good overall estimate of snail numbers within each snail habitat but is biased toward the collection of larger snails (Heppleston, 1972; Smith, 1981). The quadrant collection permits a thorough examination within a smaller more suitable microhabitat, facilitates the collection of snails hidden within the vegetation and detritus, and helps to correct for the bias of the timed collection toward larger snails. The quadrant collection site was not chosen at random but rather was selected from an area of the habitat observed to have a high density of snails. If quadrant sites were selected at random, an area devoid of snails might be chosen at a time when clusters of snails were present. This would give a false reading of liver fluke transmission potential.

Snail population data. All snails collected from an individual ranch were counted and all snails (or 184 snails, whichever was less) were measured to the nearest millimeter (rounding upward). Snails 5 mm or larger were categorized as mature (capable of reproduction) and snails 4 mm or less were categorized as juvenile (Olsen, 1947).

Determination of snail infection prevalence. During the second year of the study (May 1993 to April 1994), snails collected from all ranches with the exception of the University of Florida Dairy Research Unit (DRU) were assayed for infection with larval stages of *F. hepatica* using a DNA probe assay (see chapters 3 & 4). Snails from the DRU were not assayed because liver flukes are not known to be endemic in the herd. Only snails collected from the Brighton Seminole Indian Reservation in year one were assayed for infection.

Determination of herd infection prevalence with *F. hepatica*. Fecal samples (20 to 25 per collection) were collected from cattle of all ranches in this study with the exception of the University of Florida Dairy Research Unit. Fresh feces (still warm if available) were picked up off the ground, brought back to the laboratory, and stored at 4°C. Two gram samples were processed using a modified sedimentation technique (Flukefinder®, Visual Difference, Moscow,ID) and eggs of *F. hepatica* and *Paramphistomum* spp. were counted. Because most samples were collected shortly after being deposited on pasture, and the number of cows on pasture was much larger than the number of samples taken, it is likely that each sample was from a different animal. Large fecal pats were preferentially selected so that most samples were from cows rather than calves. Fecal samples were collected at irregular intervals because cattle frequently were not present

on the pastures and only limited refrigerator space was available to store samples prior to processing.

Tracer calf study. Because snails were found on the Brighton Seminole Indian Reservation during the summer of 1992, a small tracer study was done in the summer of 1993 to determine if summer transmission would occur. Three calves raised in a fluke-free location in northern Florida were transported to the Brighton Seminole Indian Reservation pasture on 7-3-93. One calf was a Hereford-Holstein cross (448 lb.), one was an Angus-Holstein cross (443 lb.) and one was mostly Holstein (750 lb.). These calves were allowed to graze with the rest of the herd on this pasture for the next 3 months. Calves were removed from the pasture on 10-2-93 and held in confinement until 11-22-93 when they were slaughtered and necropsied. At necropsy, livers with gall bladders were recovered and brought back to the laboratory for processing. Bile ducts were opened with scissors as far into the liver parenchyma as possible. The liver was then cut into 1 in. thick slices and each slice was manually squeezed. Liver slices were then placed in warm normal saline and soaked overnight at room temperature. The next morning, liver slices were squeezed again, thoroughly rinsed and discarded. The overnight soak solution and rinsings were poured through a #60 mesh screen and material retained by the screen was examined. Individual gall bladders were sliced open, rinsed with tap water and the bile with rinsings was poured through a 38μ mesh

screen to recover fluke eggs. Any eggs present were recovered and counted.

Weather data and calculation of potential evapotranspiration (PET) and water budgets. Daily data was obtained from 6 NOAA weather stations in Florida from January 1992 to February 1994. Weather stations were selected based on their proximity to research sites: northern Florida - Gainesville Municipal Airport (02-3326), Alachua County and St. Augustine WFOY (02-7826), St. John's County; central Florida - Mountain Lake (04-5973), Polk County and Melbourne WSO (04-5612), Brevard County; southern Florida - Archbold Biological Station (04-0236), Highlands County and Okeechobee HRCN Gate 6 (04-6485), Okeechobee County. Thirty year data (1951 to 1980) for average monthly rainfall and temperature was obtained from published data (National Oceanic and Atmospheric Administration, 1982) for the same weather stations except for Okeechobee HRCN Gate 6, St. Augustine WFOY, and Gainesville Municipal Airport for which data was not available. Thirty year data was available for Gainesville 2 WSW and this was used for 30 year calculations. Since data from Okeechobee was not available, data from Stuart, FL was used to calculate a second 30 year PET for southern Florida. Stuart and Okeechobee have the same latitude so solar radiation and daylight length are the also the same.

Potential evapotranspiration (PET) was calculated using both the Thornthwaite method (Thornthwaite, 1944) and the

Stephens-Stewart method (Stephens & Stewart, 1963). The Stephens-Stewart method was chosen because it is simple to calculate and along with the Penman method is the best predictor of evapotranspiration under Florida conditions (Jones, Allen, Shih, Rogers, Hammond, Smajstrala, & Martsolf, 1984). The Thornthwaite method was chosen because it is simple to calculate and was used by Malone et al. (1987) to develop a fascioliasis prediction model. The Stephens-Stewart method uses average monthly temperature and average monthly solar radiation to calculate PET.[3] The Thornthwaite method uses average monthly temperature and hours of daylight to calculate PET.[4] For both methods, the average daily temperature data for each station from January 1992 to February 1994 was used to calculate the average monthly temperature data used as input. Monthly solar radiation data were estimated from published tables using the same latitude data. The daylight data was estimated from published tables

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[3] $PET = 0.01476\ (T_m + 4.905)\ MR_s/\lambda$

where: PET = monthly potential evapotranspiration in mm
MR_s = monthly solar radiation, cal/cm^2
λ = latent heat of vaporization, $59.59 - 0.055\ T_m$, cal/cm^2·mm
T_m = mean monthly temperature in °C

[4] $PET = 16\ L_d[(10\ T_m)/I]^a$

where: PET = 30-day estimate of evapotranspiration in mm
L_d = daytime hours divided by 12
i = $(T_m/5)^{1.514}$
T_m = mean monthly temperature in °C
I = sum of i for all 12 months
a = $(6.75 \times 10^{-7}\ I^3) - (7.71 \times 10^5\ I^2) + (0.01792\ I) + 0.49239$

(Jones et al., 1984) using the latitude data presented in the NOAA publications. Thirty year average PET was calculated using 30 year average monthly temperature data.

Water budgets for individual weather stations were calculated using data from January 1992 to February 1994 and 30 year data. Water budgets based on 30 year average data were calculated using 30 year average monthly precipitation data, estimated 30 year PET and a soil moisture storage estimate of 6 inches (150 mm) (Mather, 1978). Water budgets for January 1992 to February 1994 were calculated using monthly PET values, actual monthly rainfall, and a soil moisture storage estimate of 150 mm. For calculation of 30 year average water budgets, available soil moisture in January was determined empirically. Initially, the water budget was determined starting with no available soil moisture in January. If available soil moisture reached capacity (6 in) in some later month, then the soil moisture available after December was used to recalculate the water budget beginning in January. If available soil moisture never reached capacity, then the water budget was recalculated beginning with the first month of a series of consecutive wet months. The water budget was then calculated for the remainder of the year and for another 12 months. As previously, the soil moisture available after December was then used to recalculate the water budget beginning in January. To begin calculation of water budgets in January 1992, the calculated 30 year average

values for available soil moisture in January was used since the true value was unknown.

Results

Northern Florida

University of Florida Dairy Research Unit (DRU), Hague, Alachua County, Florida

The DRU is a dairy cattle research facility of the University of Florida located in north central Florida. There were no recent reports of cattle on this facility being infected with liver flukes (based on personal communication with the farm manager). *Fossaria* habitats were found scattered around the eastern half of the farm in ditches, culverts, and around a drinking pond. Two *Fossaria* habitats in separate pastures were identified and marked for study.

Snail population data

Site 1 (Figure 5-1a & 5-1b). The main portion of this habitat was formed by a small drinking pond with a thick muck bottom. Extending the size of the habitat during the wetter times of the year was an adjacent drainage ditch also with a muck bottom. Snails were usually found along the muddy edges of the pond and/or along the edges of the ditch. The habitat was located in a pasture used to maintain dry cows. Other snail species observed in this habitat were *Succinea* and *Physella*.

This site was first visited for the purpose of data collection on 5-1-92. The pond was filled with water and a moderate sized population of snails consisting of approximately equal numbers of mature and immature snails were found in wet mud along the pond edge. Low rainfall and high temperatures in May caused the pond to dry and snails could no longer be found. June was a very wet month with above average rainfall and on 6-26-92 the pond was once again filled with water although only 3 snails were collected. The pond remained filled with water for the remainder of the summer but snails were not found during this period (except for a single 5 mm snail on 9-7-92). During September a small number of snails reemerged from aestivation as indicated by data on 9-26-92. On that date a large cluster of snails was observed for the first time since May 1st. However, a search of the habitat revealed only a single cluster consisting of both mature and immature snails and during the timed search only 3 mature snails were found. By the next collection on 10-16-92, a large dispersed population consisting of both mature and immature snails were present. Snails were found through the remainder of the autumn, winter, and spring, until 5-13-93 when the pond once again dried. Summer 93 was drier than normal and for most of this season the pond habitat remained dry or moist and no snails were found. An exception to this was on 7-11-93 when following a period of high rainfall, a small number of snails were scattered among the habitat. Cool

and wet weather in October caused the pond to once again fill with water and the habitat remained very wet throughout the remainder of the autumn and winter. However, only very small numbers of snails were found until 2-13-94 when an abundant and dispersed population of medium and large snails was observed. It was also noted at this time that large numbers of *Fossaria* were seen at many different locations around the DRU. Large numbers of mature snails were also found at the next collection but by 4-8-94 the pond had once again dried due to the spring drought that occurs in most years. This situation did not change on the last ranch visit of the study on 4-27-94.

Site 2 (Figure 5-1c & 5-1d). Site 2 data was obtained from 1 of 2 separate habitats in the same field, approximately 300 feet apart. The site initially designated as site 2 was in a muddy area of a wet ditch formed by tractor ruts. This ditch was fed by runoff from a pond which kept it artificially wet. Very wet conditions during summer 92 prevented this area from being mowed. These conditions produced a great deal of vegetation growth that eventually caused the habitat to essentially disappear by September i.e. no mud was present and snails could no longer be found. Therefore, a new habitat in the same field with similar characteristics was selected for study. This site also was formed by tractor ruts in a low-lying wet area. Data from this new site was collected for the first time on 9-25-92 and then for the remainder of the study.

Cows were never present in this field, however, the habitat area was occasionally disrupted by tractors which helped maintain muddy conditions. *Succinea* and *Physella* were also found in these habitats.

On 5-1-92 at the time of the first collection, the habitat area was wet and large numbers of mostly immature snails were present . By the next collection on 5-19-92 the habitat area was considerably drier although the mud was sufficiently moist for large numbers of snails to remain. The May dry spell that caused the soil at site 1 to become completely dried by 6-2-92 also caused the surface soil at site 2 to dry and no snails were found. However, because site 2 was a wetter location due to the water runoff it received, the soil just below the surface remained moist. Unlike site 1 where aestivating snails did not reappear when habitats became wet following the onset of heavy summer rains in June, large numbers of snails were found at site 2 on 6-26-92. During July and August heavy vegetation growth in and around the ditch provided shade to the areas of habitat still maintaining snail populations. During this time most snails were found in wet mud along the edges of the tractor ruts that were shaded. By 9-7-92 the habitat had become so overgrown with vegetation that the area was no longer favorable to snails and only a few were found. At the next collection on 9-25-92 the site had reached a point where it could no longer be studied and an alternate site was identified. The new site

was selected based upon its similarity to the initial site i.e. muddy area formed by tractor ruts in a wet lowlying area and the abundance of snails found there. Numbers of snails found at this site decreased over the next few collections and remained low through the winter and spring. Snails disappeared once again with the onset of spring drought (5-13-93) but unlike site 1, the soil at site 2 never fully dried. In early July heavy rains moistened the soil sufficiently for snails to become active once again. On 7-11-93 numerous, mostly medium size (4 & 5 mm) *Fossaria* were found scattered throughout the habitat. During the next 6 weeks, there was very heavy vegetation growth that shaded the entire site. On 8-1-93 vegetation growth was so thick that tractor ruts could no longer be seen and only a few snails were collected. By the next collection (8-23-93) the site had become completely overgrown with thick vegetation to the point where no areas of open soil were present. The site also was dry and no snails were found. The next 2 scheduled visits were missed but September was such a dry month that it is a certainty that no snails were present during this period. Relatively cool and wet weather in October caused vegetation growth to slow and the habitat to become wet once again. In addition the field was mowed leaving fresh deep muddy tractor ruts and snails were once again found on 10-24-93. The habitat remained wet throughout the remainder of the autumn, winter and early spring. Frequently the entire habitat was submerged and

snails could only be collected using a strainer. During January and February a large proportion of the snails collected were immature indicating that this was a period of reproduction. However, during March and April very few immature snails were collected. On the last collection of the study (4-28-94), the habitat had once again dried and no snails were found.

H. E. Wolfe Ranch, St. Augustine, St. Johns County, Florida

This ranch is located in north eastern Florida, about 15 miles from the Atlantic coast and about 2 miles east of the St. Johns River. The ranch covers 4,600 acres and maintains approximately 1100 brood cows. The ranch has a history of problems with fascioliasis. *Fossaria* habitats were found in drainage ditches along the south eastern portion of the ranch. Two of these habitats, on adjacent pastures (about 300 yds apart) were identified and marked for study. Other snail species found were *Physella* and *Succinea.*

Snail population data

Site 1. During an initial visit to this ranch on 3-24-92, a shallow drainage ditch was identified that had substantial numbers of *Fossaria* dispersed over a large area. This ditch ran parallel to a gravel road that contained a large amount of cochina shell. Two separate sites along this ditch were marked for study. However, by the time of the first collection on 4-29-92 the ditch had already become quite dry and no snails were found. It was decided at this time

that this ditch represented only one distinct site and another study site (site 2) should be identified when snails returned to pasture.

With the onset of heavy summer rains in June, the ditch began to become overgrown with vegetation. On 6-28-92 the ditch was once again wet and small numbers of *Fossaria* were found. As the summer progressed the ditch remained filled with water and became completely covered with vegetation i.e. no mud habitat was present. No *Fossaria* were found again until 12-18-92 despite the fact that the ditch remained wet through the entire summer and autumn. Small numbers of snails were found during the winter and spring of 1993 until the onset of spring drought. On 5-13-93 the habitat had become dry and no snails were found. This site remained quite dry throughout the remainder of the spring and summer as rainfall was well below normal. October was uncharacteristically wet and by 10-24-93 the ditch was once again filled with water. The ditch remained wet throughout the autumn and winter but no snails were found. During this time other areas of the same pasture were also searched but *Fossaria* were not found. On 3-8-94 *Fossaria* were finally present again but by the next collection on 4-8-94 the ditch had become fairly dry and snails could not be found.

Site 2 (Figure 5-2c & 5-2d). At the start of the study on 4-29-92, it was determined that the ditch serving as study site 1 did not adequately serve the purpose of 2 distinct

study sites. Therefore, a second study site was searched for but no *Fossaria* were found anywhere on the ranch until 10-16-92. On that date, large numbers of mostly mature snails were found dispersed along a muddy area of a wide shallow drainage ditch in a pasture adjacent to site 1 (about 300 yds. away). This area had been dry in the spring and early summer and then became covered with vegetation after the onset of summer rains. Heavy rains in September and October together with cooler weather that slowed pasture vegetation growth enabled the cows grazing this pasture to disrupt the plant cover and create an optimal wet muddy habitat for snails. The previous ranch visit was missed due to heavy rains (scheduled for 9-26-92) so the precise time of when the snails reemerged from aestivation is not certain. However, since the majority of snails collected were mature, it is probable that they had only recently reemerged i.e. in past 2 weeks or so.

At the next collection on 11-8-92 the habitat had dried considerably. Only a small number of *Fossaria* were found scattered along the surface of the mud and many shells of dead *Fossaria* were also present. On 11-28-92 the habitat was flooded from heavy rains of the previous 2 days so that most of the habitat was under a substantial amount of water. Large shells of dead *Fossaria* were found scattered along the edge of the ditch but the only live snails found were in a small muddy depression that contained a large number of 1 and 2 mm snails. At the next collection on 12-18-82, an extraordinary scene was

observed. The habitat had dried slightly and the mud was now covered by a thin layer of water with a thick carpet of algae covering the water surface. On top of the algae was an incredible number of snails; up to 25 were counted in one square inch. No other time in the course of this 2-year study was such an abundance of snails recorded (870 in a single 0.1 m^2 quadrant). All of these snails were immature, thus this represented a new generation of snails. A sizable population of snails persisted through the winter and by 3-16-93 a large number of very large snails (100% ≥ 7 mm) were found. Surprisingly, on the next visit (4-3-93) no snails were found although the habitat continued to look ideal for snails. The reason for this is not known. However, these probably died from age related natural causes since this population was made up of uniformly large snails. By the time of the next visit on 5-13-93, hot and dry weather had caused the habitat had become dry. The habitat remained dry throughout most of the summer and no snails were found. On 10-24-93, the ditch was once again filled with water as a result of heavy October rainfall but no snails were found. One snail was found on 12-5-93 and during the remainder of the autumn and winter only the occasional snail was found. Finally on 3-8-94, large numbers of *Fossaria* were present once again. However, by the next collection on 4-8-92, the habitat had dried and no snails were found. This situation did not change on the last ranch visit of the study on 4-27-94.

Herd infection prevalence with *F. hepatica*

On 8-3-92 the herd prevalence with Fasciola hepatica was 84% (ESI=2.3, n=25) even though the entire herd was treated with Ivomec-F® (Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey) on 4-30-92 (Table 5-1). The efficacy of Ivomec-F® against mature flukes (flukes > 12 weeks) is greater than 97% (Kaplan, 1994), therefore these infections resulted from metacercariae acquired after February 1st. Since snails had already entered into aestivation by late April, and metacercariae do not survive for long under hot, dry conditions (Olsen, 1947), fluke transmission would have ended by May. On 12-18-92 herd prevalence decreased to 48% (n=25) but the egg shedding index (2.1) was about the same as on 8-3-92. Any new fluke infections acquired by cattle in the autumn would not be sufficiently mature to produce eggs by 12-18-92, therefore these infections also resulted from metacercariae acquired in the spring. The decrease in prevalence could be due mixing of cow groups which is done periodically on this ranch. On 6-2-93 the herd prevalence was 40% (n=20) but the egg shedding index was 14, a value that was the second greatest of the entire study. It is interesting to note that cows were treated with Ivomec-F® on 5-1-93, therefore just as in 1992, these infections resulted from metacercariae acquired after February 1st. This is indicative of heavy fluke transmission during the late winter and spring; a situation that would be predicted based on snail population data. Cows

were again treated with Ivomec-F® on 10-1-93 and on 4-27-94 the herd infection prevalence was 0% (n=20). This is indicative of a lack of fluke transmission the preceding autumn and winter; a situation that also would be predicted based on the snail population data.

Snail infection prevalence with larval stages of *F. hepatica*

During the first year of the study when large numbers of snails and high levels of transmission was occurring, snails were not assayed for infection with larval stages of *F. hepatica*. During the second year of the study, few snails were found. On 3-8-94, the only collection of year-2 in which large numbers of snails were present, one infected snail was detected for a point prevalence of 0.52% (1/192). Overall for the year (May 1993 to April 1994), 246 snails were assayed for infection and 1 snail was positive for a prevalence of 0.41%.

Central Florida

Deseret Ranch, Deer Park, Brevard County, Florida

This ranch, one of the largest in Florida, is located in east central Florida and has over 300,000 acres in beef and citrus production. The pastures that were studied on this ranch are part of unit 13; a 7,000 acre unit divided into 38 pastures and grazed by about 1,500 cows. Unit 13 is located about 30 miles from the Atlantic coast and abuts the western shore of Sawgrass Lake. Cows were frequently rotated through different pastures to maximize forage production. *Fossaria*

habitats were found in numerous drainage ditches at points used by cows as crossing spots. These cattle crossing areas had shallower banks than the surrounding ditch and tended to have a large amount of open mud surface.

Initially 2 sites were identified for study in the same ditch of pasture 27 (about 200 yds apart). Soon after starting this study cows were removed from this pasture for an extended period of time. The lack of cattle activity caused the nature of the snail habitats to change i.e. become covered with vegetation and develop steeper banks. As a result, snail populations did not reappear in the autumn as would be expected based on snail reappearance patterns seen in similar habitats at other ranches. Because of this an alternate site (new site 2) was identified on pasture 26 which was adjacent to pasture 27 but separated by a small drainage canal. Because the 2 sites originally marked for study in pasture 27 did not differ in physiographics or in observed snail population trends, data is reported for only 1 of these sites as site 1. Site 2 data is reported from snail habitats in pasture 26 only. Other snail species found were *Physella*, *Succinea*, and *Planorbella*.

Snail population data

Site 1. Prior to the start of the study on 3-2-92, large numbers of *Fossaria* were found at virtually all cattle crossings of a series of parallel drainage ditches in pasture 27. Two separate sites (cow crossings) along one of the

ditches were selected for study, however, data reported here is from only one of those sites.

At the start of the study on 4-23-92 the habitat contained a large amount of wet mud, however, the area was considerably drier than in February. Large numbers of mostly immature snails were found. By the next visit (5-14-92) the site had dried and no snails were found. Hot dry weather persisted for the next few weeks and on the next visit (6-3-92) the soil in the ditch was extremely dry and sandy. Heavy June rains caused the ditch to become filled with water and by the next visit on 6-24-92, the banks of the ditch had become covered with vegetation. This produced an environment where very little mud was present; the previous snail habitat was now either covered with vegetation or under water and no snails were found. By the next visit on 7-15-92, vegetation had grown so extensively that habitat markers could not be seen and the area looked so different that it was quite difficult to find the selected study sites. The ditch continued to be filled with water and no snails were found. A heavy storm prevented an August collection and on 9-2-92 a few snails were found for the first time since the spring. The ditch remained filled with water and covered by vegetation. This general pattern i.e. few snails, water filled ditch, extensive vegetation cover, continued throughout the autumn. No cows were on this pasture during this period. Because no cows were present, the "cow crossing" areas with

trampled, muddy conditions no longer existed on this pasture. This resulted in a situation where optimal snail habitat was no longer present in this ditch and as such few snails were found. December and early January visits were missed. On 1-28-93 the situation had not changed, however, a single quadrant of immature snails was found. It was decided at this point to locate an alternative study site (new site 2) because the absence of cows on pasture 31 did not permit snail habitats to return to a state supportive of *Fossaria* populations.

On 2-16-93 a dispersed population of both mature and immature *Fossaria* was present for the first time since April 1992 although the area of mud habitat remained small. A similar situation was seen on the next collection on 3-9-93. On 3-30-93 no snails were found although the ditch remained wet. The April collection was missed and by 5-11-93 the ditch had become extremely dry with no possibility of snails being present. The ditch remained dry through the summer and no snails were found. On 10-5-93 the ditch was once again filled with water but with no cows on the pasture the "cow crossing" areas originally designated as snail habitats no longer existed. On 10-25-93 no snails were found and it was decided that this site was no longer worthy of study and visits to this site were terminated.

Site 2 (Figure 5-2a & 5-2b). This site in pasture 26 was visited for the first time on 1-28-93. Large populations of

Fossaria were found in several spots along a large, shallow drainage ditch. All of these *Fossaria* habitats clearly were locations used by cows to cross the ditch. Large numbers of mostly immature snails were present but collection was difficult because most snails were under water. The area remained wet and large numbers of snails were found throughout the remainder of the winter and spring until 5-11-93 when the ditch became dry and snails were no longer present.

The ditch was once again wet on 6-3-93 and although no snails were found in the same spot as previous collections, about 100 yds down the ditch a large number of both mature and immature snails were found. In this same area on 6-22-93, very large numbers of mostly mature snails were found. By the next collection on 7-12-93, the ditch had dried considerably and only a few snails were collected. On 8-13-93 the ditch was once again filled with water and snail habitats were essentially submerged. No snails were found, however. The very high rate of evapotranspiration on pastures in Florida during the summer can rapidly cause areas to dry if rainfall ceases for short periods. This was seen in August as the ditch was totally dry again (8-24-93) only 3 weeks after being completely filled with water. Snails were found again on 10-5-93 indicating that they had reemerged from aestivation in late September or early October. Very large numbers of snails were found during the remainder of the autumn and on the 12-6-93 collection the majority of snails were immature suggesting

that a major reproductive effort and generation turnover occurred during November. By 12-29-93, however, the ditch had dried once again and no snails were found.

The ditch remained fairly dry during the rest of the winter and no snails were found until 3-8-94 when the ditch once again became filled with water and large numbers of immature snails were found beneath the water level. At the next 2 collections (last 2 collections of the study) fairly large numbers of mature *Fossaria* were found. It is interesting to note that on 2-8-94, when site 2 was too dry to support active snail populations, an area in a different pasture receiving runoff from a free flowing artesian well was observed to contained very large numbers of *Fossaria*.

Herd infection prevalence with *F. hepatica*

Cattle from pasture 27 were never sampled to determine herd prevalence because cows were rarely present. Cows from pasture 26 were sampled only once on 4-5-94 (Table 5-1). This was because cows frequently were not present on the pasture, and by chance, on those days when sampling supplies were brought no cows were there to collect samples from. On 4-5-94 herd prevalence was 50% (ESI=0.6, n=20). Cows were treated once annually in September with Albendazole.

Snail infection prevalence with larval stages of *F. hepatica*

One infected snail was detected on 6-3-93, 3 infected snails were detected on 10-5-93, and 2 infected snails were detected on 4-26-94. Overall 6 snails were infected out of a

total of 784 snails assayed for a yearly prevalence of 0.77%. Although this data does not appear to yield much of importance when presented this way, when examined a little more closely, this data reveals a few interesting points. The infected snails on 10-5-93 (3/32, 9.4% point prevalence) were from a population of snails that had only recently emerged from aestivation and no cows were present on pasture at the time of that visit. Two of the 3 infections were scored as 3+, therefore these infections were probably greater than 21 days old. It is unlikely but not impossible that these snails were present on the pasture surface 21 days earlier. Therefore, either these snails were infected in the spring or early summer and survived aestivation as infected snails (most likely), or they were infected almost immediately upon returning to the pasture surface following reemergence from aestivation (less likely).

All cows in this unit were treated with albendazole in September and cows were only present on pasture 26 at the time of snail collections on 2 occasions after this date; 10-25-93 and 4-5-94. No infected snails were detected on 10-25-93, but since cows were treated in September, they would not be shedding fluke eggs onto pasture and infected snails would not be expected to be found during this period. No cows were seen on the pasture during the remainder of the autumn or winter. Cows were present once again on 4-5-94 and 2 infected snails (2/21, 9.5% point prevalence) were found at the next

collection on 4-25-94. Although it is possible that cows were occasionally present and not seen on this 332 acre pasture, and that cows were present between collections but removed prior to ranch visits, it is most probable that for the majority of this period that no cows were present. Therefore, the low snail infection prevalence determined for this pasture is probably an artifact resulting from an absence of cows and subsequent lack of pasture contamination with fluke eggs. Unfortunately, dates of cattle rotations between pastures was not available from the unit manager.

Creek Ranch, Lake Hatchenehaw, Polk County, Florida

This ranch is located in central Florida about 8 miles east of the town of Lake Hamilton and borders the western shore of Lake Hatchenehaw. The first visit to this ranch was on 10-13-92, approximately 6 months into the study. The reason for this delay was that a second central Florida study site could not be identified prior to the start of the study. Several central Florida ranches with a history of fascioliasis were visited during the spring of 1992, but no *Fossaria* habitats were found. Spring drought and summer aestivation then prevented a second study site from being identified until the autumn.

Two sites were identified for study on the same 60 acre pasture, several hundred yds. apart. The pasture was drained by a series of shallow drainage ditches and populations of *Fossaria* were found at several cow crossing locations. Site

1 was selected from one of these spots. Site 2 was a low lying muddy area about 40 ft from a free-flowing well, although runoff from the well drained into a ditch and did not directly supply this location with water. Muddy conditions in this area were maintained by cattle activity as evidenced from the numerous hoof prints that were usually present.

Snail population data

Site 1 (Figure 5-3a & 5-3b). On the first collection at this site on 10-13-92, a small population of very large *Fossaria* were present suggesting that they recently emerged from aestivation. When these snails were brought back to the laboratory to be measured and counted, greater than 20 egg packets were recovered. These spring generation snails were thus producing large numbers of eggs that would form the new autumn generation of snails. However, by the next collection on 11-4-92 the ditch had dried and no snails were found. The ditch remained dry during November but December rains once again filled the ditch with water and a mixed population of both immature and mature snails were found on 12-16-92. Wet, muddy conditions persisted during the winter and spring and a large population of *Fossaria* was present throughout this period.

Hot and dry weather in May caused the ditch to dry and on 6-3-93, snails were no longer present. Summer 1993 had below normal rainfall and the ditch remained dry throughout the summer. On 10-5-93, the ditch had once again become filled

with water but no snails were found. However, by 10-25-93 the ditch had again dried and the ditch remained dry during the remainder of the autumn. Following significant January rainfall, the ditch was again filled with water on 1-19-94 but no snails were found. On 2-8-94 a dispersed population of snails were present for the first time since the preceding May. Virtually all of the snails that were collected were immature. By the next collection on 3-7-94 the habitat area was almost completely submerged but a fair number of mature snails were found dispersed along the muddy edges. A month later on 4-5-94 the ditch was once again dry and no snails were found. On the final collection of the study (4-25-94) the ditch remained dry.

Site 2 (Figure 5-3c & 5-3d). On the first collection at this site on 10-13-92, a very large number of Fossaria were found distributed throughout the habitat. This level of snail abundance is typical of an actively reproducing *Fossaria* population. Recently emerged (aestivating) populations tend to be much smaller in size and more dispersed (as seen at site 1 on this date). *Fossaria* at this site were therefore actively reproducing for at least several weeks prior to this visit. Three weeks later the area had dried considerably and only a few snails were found in the moist bottoms of hoof prints. On the next visit the habitat was once again wet but it appeared that the area had only recently become wet and prior to that had been fairly dry. No snails were found. On

12-16-92, large numbers of mostly immature snails were found in the bottom of virtually every hoof print in the vicinity.

For the next 6 months, throughout the entire winter and spring, this area of pasture remained wet and large numbers of snails were present. It is interesting to note that throughout this period, the majority of snails collected were immature and only very few snails ≥ 6 mm were found. On 6-22-93 the habitat was too dry for snails but on 7-12-93 the habitat was sufficiently moist and a moderate number of *Fossaria* were found. Most of the mud in this habitat was covered with vegetation by this time and snails were found in the moist mud beneath the vegetation. This collection was the first at this site where a majority of the snails were mature. At the time of the next collection on 8-3-93, the area was completely overgrown with vegetation except for portions of several tractor ruts. In one of these ruts a small cluster of immature *Fossaria* was found. The area remained fairly dry throughout the autumn but never dryed out; the soil always maintained a degree of moisture. No snails were found during this period except for a small number of immatures on 10-25-93. On 1-19-94 the habitat was submerged from recent heavy rains and small numbers of *Fossaria* were observed beneath the water level. By the next collection on 2-8-94, the area had dried considerably, but the mud was sufficiently moist to support a large number of snails, most of which were mature. The habitat remained moist on the next visit (3-7-94) and a

large number of immature snails were present. A small number of snails were found on the next visit (4-5-94) and on the last visit of the study on 4-25-94, a large number of mostly immature snails were found distributed throughout the habitat.

Herd infection prevalence with *F. hepatica*

Cows were sampled only once on 2-8-94 (Table 5-1). Herd prevalence was 5% (ESI=0.003, n=20) with only 1 of 20 samples positive for *F. hepatica* eggs. Cows were treated once annually in November with Albendazole.

Snail infection prevalence with larval stages of *F. hepatica*

Only 2 of 470 snails assayed were infected for a prevalence of 0.43%. One infected snails was detected on 2-8-94 and one on 4-5-94. This low prevalence is reasonable based on snail population trends and treatment history. After the 5-11-94 collection, no snails were present at site 1 until 2-8-94. Although small numbers of snails were present on occasion at site 2, the area never was wet. Without a surface film of water it is unlikely that snails would become infected. In addition, all cows were treated with albendazole in November. Therefore, cows would be essentially free of flukes after this time and little to no new transmission would be expected to occur during the late autumn or early winter since the snail habitats remained dry.

Southern Florida

Brighton Seminole Indian Reservation, (Glades County), Florida

The Seminole Indian Reservation is located along the northwestern edge of Lake Okeechobee north and east of Lakeport. The Seminole Tribe has a major cattle operation on this reservation with approximately 45,000 acres of pasture and greater than 5,000 head of cows. The pasture used for this study (Osceola pasture) was in the southern portion of the Reservation, approximately 2 miles west of the Harvey Pond Canal and 3 miles north of Lake Okeechobee. This pasture was approximately 200 acres in size with an average of 88 cows grazing it at any one time. Cows were treated annually in March with albendazole.

Two sites were identified for study. Site 1 was a drinking pond with a thick muck bottom and contained little to no vegetation. Snails were found in and around the pond; especially along the muddy edges, in hoof prints around the wet areas bordering the pond and in the cow paths leading up to the pond. This location supported the largest and most consistent population of *Fossaria* that was observed anywhere during this study. The pond was in a very low area of the pasture and remained wet for most of the study. Only with sustained drought did this site begin to dry. Site 2 was located on the other end of the pasture, approximately 500 yds away. This site was formed by a low, swampy area referred to as a flag pond. Just as with site 1, snails were found along

the muddy edges of the flag pond, in hoof prints around the wet areas bordering the flag pond and in the cow paths leading up to the flag pond. This area dried much more quickly than did site 1 during periods of low rainfall. Other snails species found on this pasture were *Planorbella*, *Succinea*, *Physella*, *Eucladyna* and *Pomacea paludosa*.

Snail population data

Site 1 (Figure 5-4a & 5-4b). On the first collection at this site on 4-24-92, the pond was not holding any water although the mud bottom of the pond was still moist. A moderate number of immature *Fossaria* were found distributed along the surface of the mud. On the next visit (5-15-92), the pond mud was a little wetter, but no standing water was present. However, very large numbers of both immature and mature snails were found along the pond bottom in hoof prints. By the next visit (6-4-92) the pond was partially filled with water and large numbers of both mature and immature snails were found in the wet mud along the water edge. On 6-25-92 a tropical depression hit this area of Florida so a collection could not be made. Overall, June was an extremely wet month with total rainfall of almost 19 inches. At the next collection on 7-16-92, the pond was filled beyond capacity and the areas around the pond were under water. Snails were seen dispersed under the water level and a small number of mature snails were collected in the wet muddy areas of the cow paths leading away from the pond. The entire habitat area around

the pond remained essentially submerged for the remainder of the summer and into the early autumn. During this period very little exposed mud was present. The small amount of mud that was present at any given collection was due to cattle activity that would turn up tufts of turf creating a small mound of mud. Small numbers of snails were found throughout this time mostly in these tufts of mud. The fact that only small numbers of snails were found does not preclude the possibility that a much larger population was present under the water level.

By 11-5-92 the pasture had dried considerably and the habitat had returned to a more normal state. The pond remained filled with water, but the pond edge and surrounding area consisted of mostly wet mud. Large numbers of mostly mature snails were found along the edge of the pond. The remainder of the pasture was fairly dry at this time and shallow drainage ditches that had been filled with water during the summer and early autumn were now dry. A major reproductive effort occurred in November and on 11-24-92 a very large number of immature snails were found lining the wet muddy hoof depressions in the area immediately surrounding the pond. The habitat remained wet through the autumn, winter and spring and a large population of *Fossaria* was present during this period.

Another major reproductive effort occurred during April and on the 5-12-93 collection, very large numbers of mostly

immature *Fossaria* were found along the edge of the pond. On the next visit on 6-4-93, no snails were found even though the habitat area was wet. However, May was an extremely dry month and it appeared that the habitat had become fairly dry prior to heavy rains that fell during the first week of June. By the next visit snails were found again, mostly in wet depressions several ft from the pond edge. On 7-13-93 the habitat area had again dried substantially but the pond still was still holding a small amount of water and a large area of very wet muck was present on the pond bottom. Although this environment looked ideal for snails (similar to 5-15-92 when very large numbers of Fossaria were present), none were found. Rainfall during the summer of 1993 was well below normal and by August the habitat area was extremely dry.

The habitat area was wet once again on 10-6-93 following significant September rainfall, but no snails were found. On the next visit (10-26-93) the pond was filled to capacity and little mud was left uncovered by water or vegetation. Still no snails were found, however. Finally on 11-16-93, *Fossaria* had reemerged from aestivation and large numbers of mostly medium size snails (3 to 5 mm) were found all along the pond edge. During the remainder of the autumn, winter and spring the habitat remained either wet or submerged and large numbers of *Fossaria* were found. An exception was on 12-30-93 when for a reason that remains a mystery, no snails were found.

January 1993 was an extremely wet month and during the January and February visits virtually all snails collected were found under the water. On 4-6-94, an extraordinary scene existed at this habitat site. An incredible number of snails were present--the mud around the edge of the pond was virtually carpeted with snails. A record 465 snails were collected in a single quadrant. Although this is less than the 870 recorded on 12-18-92 on Wolfe ranch, 93% of the snails collected here were ≥4 mm in size, whereas on the 12-18-92 collection 93% were ≤3 mm. A timed collection would not give a valid estimate of population density since snails could literally be scooped up by the handful. However, for curiosity's sake a 1 min timed search was done and 250 snails (>4 snails/sec) were collected. Three weeks later on the last visit of the study (4-26-94) very large numbers of snails were still present (although significantly fewer than on 4-6-94).

Site 2 (Figure 5-4c & 5-4d). The dynamics of *Fossaria* populations at site 2 were very similar to those at site one, so a complete narrative will not be presented. The major differences between these 2 sites were (1) populations at site 2 were less dense and (2) site 2 was more prone to drying during periods of low rainfall. Two difference worth noting are the summers of 1992 and 1993. In both years, site 2 dried and *Fossaria* disappeared from the surface sooner than on site 1. Unlike site 1, site 2 became dry in the spring of 1992 (5-15-92) forcing snails to aestivate. After site 2 became wet

with the start of the summer rains, only very few snails were found until the autumn. The next spring (on 5-12-93), when site 1 was still quite wet with a large population of *Fossaria*, site 2 was mostly dry. The only portion of the habitat that remained wet was toward the center of the flag pond where a small number of snails were found. On 6-4-93 the site was wet but clearly had been very dry prior to early June rains. For the remainder of the summer, the main portion of the habitat remained quite dry and no snails were found. Snails reappeared on 11-16-92, the same day as for site 1. However, only very few snails were found at site 2 in contrast to site 1, and large numbers of snails were not found at site 2 until 1-20-94.

Herd infection prevalence with *F. hepatica*

Fecal samples were examined 5 times during this study at approximately 6 month intervals (Table 5-1). Infection prevalence was 32% on 7-16-92, approximately 4 months after treatment with albendazole. Most of the flukes infecting the cattle at this time were thus acquired after the 1st of January 1992 (immature and not susceptible to flukicide in March) and before May (flukes old enough to produce eggs). This ranch was visited twice prior to the start of the study; on 1-24-92 and 3-18-92. On 1-24-92 when this pasture was first selected for inclusion in this study, the pasture was fairly dry and very few snails were found. On 3-18-92 the pasture was very dry, including site 1, and no snails were

found. Therefore, even at a time when there was probably little transmission occurring on this pasture, infection prevalence was still 32%. On 12-18-92 the infection prevalence had increased to 64%. Since flukes acquired after October 1st would not be producing eggs by mid-December, a considerable number of the new infections causing an increase in herd prevalence were probably acquired between May and September. Thus, it is very likely that some summer transmission occurred.

Cattle were next sampled on 5-12-93 and infection prevalence was 15%. This low prevalence was the result of treatment with albendazole in March and would represent infections acquired mostly during January and February (infections acquired later than March 1 would not be sufficiently mature to produce eggs by 5-12-93). On 11-16-93 herd infection prevalence reached 100% with an egg shedding index of 15.4 which was the greatest value for this index during the entire study. Three tracer calves were placed on this pasture for the period of 7-3-93 to 10-2-93. Necropsy on 11-22-93 revealed only 1 fluke in the 3 calves (Holstein), therefore essentially no fluke transmission occurred during the summer of 1993. Thus, this 100% prevalence represents heavy fluke transmission during the winter and Spring of 1993, especially during the months of March - June. On 4-26-94 herd prevalence was again 15% following March Treatment with albendazole.

Snail infection prevalence with larval stages of *F. hepatica*

One infected snail (0.9% point prevalence) was found on 5-12-93 at which time the snail habitat was still optimal and contained large numbers of snails (Figure 5-5). On the next collection three weeks later (6-4-93), the habitat had dried considerably and no snails were found. Three weeks later (6-23-93) the habitat was wetter and snails returned, however none of those assayed were infected. Following this collection snails were not found again until 11-16-93 when snails emerged from summer aestivation. No infected snails were found in this population of newly emerged snails. Three weeks later (12-7-93), 7 of 131 snails were infected (5.3% point prevalence) and infected snails were found continuously throughout the winter and spring with infection prevalence ranging from 2.2 to 5.2% per collection.

John Williams Ranch, Okeechobee, Okeechobee County, Florida

This 130 acre ranch is located within the city limits of Okeechobee and maintains between 80 and 150 cows. No specific management information was available, however, cows are rotated through several different pastures. The pasture used in this study was on the western portion of the ranch with entrance directly from US 441. This ranch was the last to be included in this study, with data collection beginning on 3-10-93. The reason for this delay was that a different site had been studied during the first 11 months of the study. By March, 1993, it was determined that this other ranch was a

poor choice and did not reflect a site representative of the region. The Williams ranch was therefore chosen as a new study site for the remainder of the study.

Initially 2 sites were selected for study; the muddy edges of a large drinking pond and the muddy bottom of a shallow drainage ditch. Additionally, this pasture had a wet, marshy area where *Pseudosuccinea columella* were found. This marshy area was the only location on any pasture included in this study where *P. columella* were found. Other snail species found include *Physella*, *Planorbella* and *Melanoides*. No specific treatment program for liver flukes was used on this ranch but during the time period of this study, cattle were treated twice; on 9-29-92 with Ivomec-F® and on 5-3-93 with clorsulon.

Snail population data

Site 1 (Figure 5-6). This site was in a shallow drainage ditch with a dark muddy (muck) bottom. The main portion of the habitat was a muddy area formed as a result of cattle activity but was not truly a cattle crossing area. About 50 ft down the ditch was a major cattle crossing location and *Fossaria* were frequently found there as well. This whole area of the ditch (about 60 foot length) was studied as site 1, the specific location of collection depending upon the distribution of the snail populations.

On 3-10-93 this site was visited for the first time. Large numbers of both immature and mature snails were found

dispersed along the wet mud surface. On the next visit the habitat remained wet and large numbers of mostly immature snails were present. By the next visit 6 weeks later on 5-12-93, the habitat had completely dried and snails had entered into aestivation. On 6-4-93 the ditch was wet from rains earlier in the week, but no snails were present. The ditch remained dry but was completely overgrown with vegetation on 6-23-93. Rainfall in the summer 1993 was well below normal and for the remainder of the summer the ditch remained dry and no snails were found. By October, soil in the ditch had become moist but snails still were not present. On 11-16-93 the ditch had finally become wet and a small dispersed population of medium and large *Fossaria* were present. This size and spacial distribution is typical of snails recently emerged from aestivation. At the next collection on 12-7-93, the ditch was considerably wetter than 3 weeks earlier with the length of the ditch holding water and the habitat fully saturated. Very large numbers of immature snails were present throughout the habitat area indicating that snails present 3 weeks earlier had died and a new generation of snails was established. Of greater than 200 snails collected, only 2 mature (both 8 mm) snails were recorded.

On 12-30-93 the habitat was essentially submerged and although large numbers of small snails could be seen under the water, collection was difficult. By 1-20-94, the ditch resembled a small river and for all practical purposes, no

habitat existed. A few medium sized snails were found floating upside down along the water surface as they were being carried down the ditch by the current. Under these environmental conditions a great deal of snail dispersion takes place allowing new sites to become colonized. On 2-9-94 the ditch remained filled with water but large numbers of mostly mature snails were found. Most snails present were found attached to vegetation although a fair number were found as clusters of 3 to 5 floating together in the water. The ditch remained filled with water on the next 2 visits but the cattle crossing area contained large amounts of wet mud and large numbers of both immature and mature *Fossaria* were found in this location. On the last visit of the study (4-26-94) the ditch remained wet but only a small number of snails were found.

Site 2. This habitat was formed by a drinking pond which had a large amount dark wet mud along the water edge. Snails were found only in the mud, never in the water. On 3-10-93 a moderate number of immature and mature snails were found in the wet mud near the water edge. Three weeks later a large number of mostly immature snails were found in several wet muddy depressions. By 5-12-93 a heavy layer of algae had grown on the pond surface and only a few snails were found. On the next visit no snails were found although the pond remained filled and wet mud was present along the water edge. For the remainder of the study this situation persisted i.e.

pond remained filled, wet mud was present along the water edge and no snails were found.

Herd infection prevalence with *F. hepatica*

Herd prevalence was examined 3 times at approximately 6 month intervals on 5-12-93, 11-16-93, and 4-26-93 (Table 5-1). On 5-12-93 prevalence was 63%. According to records obtained from the ranch owner, cows were treated with clorsulon on 5-3-93, only 9 days earlier. Clorsulon kills mature flukes with >99% efficacy and also kills immature bile duct stages with fairly high efficacy. Therefore, if these cattle were treated appropriately with clorsulon 9 days earlier, then it would be highly improbable that eggs would be found in 63% of samples. It is likely, therefore, that this treatment date is incorrect. On 11-16-93 prevalence fell to 20% and on 4-26-94 prevalence was only 5%. On 4-6-94, samples were taken from 2 fecal pats that were deposited in the mud at site 1. At the time these samples were collected, large numbers of snails were present in the area surrounding the fecal pats. One of these 2 samples contained eggs of *F. hepatica*. This herd prevalence trend and the fact that infected feces were found in the vicinity of snails on 4-6-94 are concordant with snail infection prevalence data (see below).

Snail infection prevalence with larval stages of *F. hepatica*

By 5-12-94 (at the beginning of the snail infection prevalence portion of the study), spring drought had caused snail habitats to dry and snails had entered into summer

aestivation. No snails were found again until 11-16-93 when snails emerged from summer aestivation. Nine of 51 snails (17.6% point prevalence) assayed from this newly emerged population of snails were infected and 8 of the 9 snails harbored infections estimated to be at least 21 days old based upon hybridization signal intensity (Figure 5-7). By the next collection (12-7-93), most of the snails present 3 weeks earlier had died and a new population of snails was present with only 2 of 218 snails being greater than 4 mm. One of these 2 large snails (both 8 mm) was infected so although the overall infection prevalence for the collection was 0.6%, 50% of the snails remaining from the spring population were infected. This pattern of snail infection strongly suggests that infected snails survived summer aestivation. No infected snails were detected in the new autumn generation and infected snails were not found again until 4-6-94 when 9 of 146 (6.2% point prevalence) were infected. On 4-26-94, 1 of 19 (5.3% point prevalence) snails was infected.

Rio Ranch, Okeechobee, Okeechobee County, Florida

This ranch was located about 10 miles due west of Okeechobee. Several free-flowing artesian wells were used on this ranch as supplementary water sources during much of the year. One of these wells fed into a series of drainage ditches where large numbers of *Fossaria* were found. Two sites in this area were selected for study.

The water flow from the well fed directly into a large, north-south directed drainage ditch. Perpendicular to this ditch was a series of small, shallow drainage ditches that were fed from runoff of this larger ditch. Directly across from the well head was one of these smaller ditches and this ditch served as site 1. Large numbers of snails were found in this ditch, most often in the area proximal to the well. The portion of site 1 nearest the well was a dark sandy area on the wet banks of the ditch. This was the only location in the entire study where snails were found in a site that did not have a surface soil composed of a dark organic (muck) mud. This site also had the largest populations of other snail species that were observed in this study. Other snail species found at this site were *Planorbella*, *Succinea*, *Physella h. hendersoni*, *Biomphalaria havanensis*, and *Melanoides tuberculatus*. Whereas *Fossaria* was the dominant snail specie at all other locations studied in this project, at this site, extraordinarily large populations of *Physella h. hendersoni*, *Biomphalaria havanensis*, and *Melanoides tuberculatus* frequently were present. Because these other snail species were so plentiful, their presence is mentioned below when describing the conditions of the habitat. A little further down the ditch (approximately 30 to 100 ft from the well), the soil turned to muck and *Fossaria* were frequently found in this area as well.

Site 2 was in a ditch that paralleled the site 1 ditch, approximately 200 ft to the south. This ditch also received runoff the large north-south ditch and had a thick muck soil layer. Cows were treated with flukicide only once during the time frame of this study; in April 1992 with Ivomec-F®.

Snail population data

Site 1 (Figure 5-8a & 5-8b). On the first visit to this site on 5-14-92, large numbers of mature *Fossaria* were found on the wet sandy banks of the ditch all along the water edge. This location was on the eastern most portion of the ditch (near the border of the large north-south ditch) about 20 ft. from the well head. By the next visit on 6-4-92, heavy rains had caused the habitat area to become flooded. Only 2 Fossaria were found and these were attached to vegetation. Large numbers of *Physella* and *Melanoides* were present, however. On 7-16-92 the water level had dropped considerably once again exposing the wet sandy banks. Large numbers of *Physella* were present but no *Fossaria* were found. Three weeks later conditions at this site were very similar and no *Fossaria* were found, although *Physella* and *Melanoides* were present.

On 9-3-92 the well was turned off and the previously defined habitat area was no longer holding water although the soil remained moist. The large N-S ditch still held a small amount of water. No *Fossaria* or *Physella* were found but a few *Succinea* were present and large numbers of *Melanoides* were

present in the area directly in front of the well head that still held water. Conditions remained the same on the next visit (well still turned off) but this time a few *Fossaria*, *Physella*, and *Planorbella* were also present. On 10-4-92 the well head was still off and no standing water was present, however, the soil in the bottom of the ditch remained wet. Small numbers of mostly mature *Fossaria* were found dispersed on the mud surface along the ditch bottom. Small numbers of *Succinea* and *Planorbella* were present and large numbers of sickly looking *Physella* and *Melanoides* covered the mud surface.

The well was turned back on at the next visit (11-5-92) and the ditches were once again filled with water. Only 1 *Fossaria* was found and only very few snails of any other specie were found. The habitat remained wet on 11-24-92, and a few immature Fossaria were found. Large numbers of *Physella* were once again present but *Melanoides* appeared to be dead. On 12-17-92, a sizeable population of *Fossaria* (mostly mature) was present again for the first time since May. By the next visit on 1-8-93, a major reproductive effort had occurred and very large numbers of mostly immature *Fossaria* were present all along the muddy ditch bottom west of the well. On the next visit 3 weeks later (1-29-93), heavy January rainfall had caused the ditches to become filled with water and the entire habitat area was under. Large numbers of large *Fossaria* could be seen under the water and a fair number of large and very

large snails (median snail size = 8 mm) were collected off of the water surface as they were floating with the water current. The flooded conditions caused difficulty in the collection of snails and therefore the number collected greatly underestimates the true number that were present.

By 2-17-93 much less water was present and a large amount of very wet mud covered the ditch. An extraordinary number of both immature and mature *Fossaria* were present; the most observed at any time on this ranch and the second most counted in a quadrant area during the entire study. Three weeks later on 3-10-93, the ditch had dried considerably and the soil was just barely moist enough to find *Fossaria*. A moderate number of snails were found dispersed over the moist mud surface but these represented only a small fraction of the population that was present on the previous visit. The well head was on at this time, but a small mound of soil had accumulated at the eastern most portion of the ditch preventing water from flowing into this ditch from the main N-S ditch. The ditch remained moist on the next visit (3-31-93) and moderate number of Fossaria were present. While the ditch west of the well head was only moist during the 2 March collections, the eastern most edge of the ditch across from the well head (where a large *Fossaria* population was present at the 5-14-92 collection) remained wet although no *Fossaria* were found there.

By 5-12-93 the ditch west of the well head had completely dried as a result of hot and dry spring weather and a lack of water flow from the well (due to the soil mound barrier that had formed). However, *Fossaria* were once again found near the well head. *Physella* were also fairly abundant in this area. All ditches were once again filled with water on 6-4-93 and a small number of *Fossaria* were found in the mud across from the well head. Heavy June rains caused the entire habitat area to become flooded and no exposed mud was present. Two Fossaria were found that had climbed onto vegetation and out of the water. *Physella*, *Biomphalaria* and *Melanoides* were present. Conditions remained essentially unchanged at the next visit (7-13-93) and several *Fossaria* were again found attached to vegetation just above the water level. By August the water level had receded and wet muddy areas were again plentiful although only 2 (8-4-93) or 1 *Fossaria* (8-25-93) were found.

Finally on 10-26-93 a fairly large population of mostly medium sized snails were once again present. These snails had most likely only recently emerged from aestivation. By the next visit on 11-16-93, a reproductive effort had occurred and large numbers of mostly immature snails were found. The habitat area remained wet for the remainder of the autumn, winter and spring and a stable population of *Fossaria* were present throughout this period. Large numbers of *Physella* were also found during this period.

Site 2 (Figure 5-8c & 5-8d). The dynamics of *Fossaria* populations at site 2 were very similar to those at site one, so a complete narrative will not be presented. The major differences between these 2 sites were (1) populations at site 2 were usually less dense and (2) in year-2, *Fossaria* populations did not return to site 2 until about 3 1/2 months after they had returned to site 1 following summer aestivation.

Herd infection prevalence with *F. hepatica*

Herd prevalence was examined only once at this ranch on 8-13-92 (Table 5-1). Of 25 samples examined none contained eggs of *F. hepatica* (0% prevalence). Cattle on this ranch were treated with Ivomec-F® just prior to the start of the study in April 1992. Treatment at this time would not have killed liver flukes acquired after January. Since liver fluke transmission is greatest during the spring, if liver flukes were enzootic on this pasture one would expect cows to be passing large numbers of eggs by August. Cattle are rotated between pastures on this ranch so it is also possible that the group of cows sampled had been grazing a pasture that did not harbor populations of *Fossaria*. However, there was no way to determine this because management records were not available. Because of this uncertainty in the data, effort was not spent to reexamine fecal samples from this pasture.

Snail infection prevalence with larval stages of *F. hepatica*

Of 1410 snails examined, only 1 infected snail was detected on 11-16-93 for a prevalence of 0.07%. This is concordant with a 0% prevalence that was determined for cows on this pasture on 8-13-92 (see above). Although this ranch had a history of fascioliasis, apparently little transmission was occurring presently.

Water Budgets for Selected Sites in Florida

Thirty year water budgets were calculated using PET values that were calculated using both the Thornthwaite and Stephens-Stewart methods using data from: Gainesville 2 WSW (02-3321), Alachua County; Melbourne WSO (04-5612), Brevard County; Mountain Lake (04-5973), Polk County; Archbold Biological Station (04-0236), Highlands County; and Stuart 1 N (06-8620), Martin County (Tables 5-2, 5-3, 5-6 to 5-9, 5-12 to 5-14; Figure 5-9 to 5-18). The 2 methods for calculating PET gave substantially different results. Using the Thornthwaite method, water deficit (e.g. AET < PET; available soil moisture insufficient to permit AET from reaching PET) never occurred at any of the 5 sites. In contrast, when PET was calculated using the Stephens-Stewart method, water budgets revealed frequent and sustained periods of water deficit at every site examined. A consistent feature of these water budgets was that April and May were the driest months of

the year. This did not change when looking at the different climatic regions of the state.

Water budgets were also calculated for the period of January 1992 to February 1994 using PET values that were calculated by both the Thornthwaite and Stephens-Stewart methods. Data was collected from six NOAA weather stations: Gainesville Municipal Airport (02-3326), Alachua County; St. Augustine WFOY (02-7826), St. John's County; Melbourne WSO (04-5612), Brevard County; Mountain Lake (04-5973), Polk County; Archbold Biological Station (04-0236), Highlands County; and Okeechobee HRCN Gate 6 (04-6485), Okeechobee County (Tables 5-2 to 5-13; Figures 5-19 to 5-30). Because the 2 methods for calculating PET gave very different results and the physiographics of each *Fossaria* habitat varied with respect to the degree that rainfall affected soil moisture, no statistical correlations were performed.

Discussion

This study was performed over a 2-year period using 7 separate research sites throughout the state of Florida. Habitats of *F. cubensis* were identified and the bionomics of snail populations located at these sites were monitored. In addition, a DNA probe assay was developed that enabled the collection of accurate and complete data on snail intermediate host infection prevalences. Using this approach, the seasonal transmission dynamics of *F. hepatica* was determined for the

state of Florida. Contrary to predictions of a computer model (Malone & Craig, 1990) seasonal transmission dynamics for northern and southern Florida were essentially the same. Transmission of liver flukes in southern Florida occurs predominantly in the winter and spring; the same exact pattern reported for northern Florida by Boyce & Courtney (1990). Whether or not any appreciable transmission occurs in the autumn will depend largely upon yearly rainfall patterns. Wet summers followed by cool and wet weather in September and October could result in significant levels of transmission occurring by December. Summer transmission will rarely occur in Florida. Although snail habitats remain wet throughout the summers of most years, the summer ecological conditions of *Fossaria* habitats are not conducive to snail activity.

Soil moisture has been shown previously to be the most important factor determining seasonal variation in liver fluke transmission when temperatures are sufficiently high to permit snail and parasite development (>10°C) (Malone et al., 1984/85; Ollerenshaw & Rowlands, 1959; Ross, 1975). In northern Florida (Gainesville), the lowest average monthly temperature is 13.6°C (January), therefore, temperatures sufficiently high for snail and parasite development occur in Florida during the entire year. Soil moisture would then be expected to be the key determinant of seasonal *F. hepatica* transmission. However, this may not be true in Florida. Florida has a hydrologic cycle that is unique among

southeastern states (Jones et al., 1984). Most of the rest of the southeast has a winter wet season that begins in November or December and extends through March or April. This is in contrast to peninsular Florida where the heaviest rainfall usually occurs during June through September or October, when rainfall exceeds evapotranspiration. Beginning in October or November, rainfall decreases and drier weather typically continues through May. During both years of this study, *Fossaria* populations were greatest from October to May. From June through September, few or no snails were found at virtually all research sites included in this study. This pattern of snail population density is thus inverse to what would be expected solely on the basis of average yearly rainfall patterns. It could be argued that data from a 2-year study are insufficient to make inferences regarding 30-year average patterns. However, rainfall patterns were quite different for the 2 years included in this study, while snail population patterns only differed slightly. This was true for northern, central and southern Florida (Figure 5-31).

Prior to this study, it was suspected that the seasonal population dynamics of *F. cubensis* in subtropical southern Florida might be different than that in temperate northern Florida. If differences did exist, then the seasonal transmission dynamics of *F. hepatica* would also differ. However, no real differences were seen in this study. This can be explained by closer examination of the Florida climate.

From November to April, northern Florida is considerably cooler than southern Florida. During this period PET for northern and southern Florida differ by about 30% (Jones et al., 1984). From May to October the climate is very similar throughout the entire state, i.e., hot and humid. Therefore, the climatic differences between northern and southern Florida occur during the time of year when snail populations are greatest. The fact that northern Florida is considerably cooler has little effect on snail bionomics because the climate is still warm enough for snails and parasites to remain active.

To help understand the effects of available soil moisture on the population dynamics of *F. cubensis* in Florida, water budgets were calculated using PET values computed by two separate methods. The Stephens-Stewart method was chosen because: (1) it is simple to calculate; (2) the only data required for calculation is available from weather stations and published tables; and (3) along with the Penman method, it is the best predictor of evapotranspiration under Florida conditions (Jones et al., 1984). The Thornthwaite method was chosen because: (1) it is simple to calculate; (2) the only data required for calculation is available from weather stations and published tables; and (3) it was used by Malone et al. (1987) to develop a fascioliasis prediction model. However, the Thornthwaite method was determined to be the

poorest predictor for PET of 5 methods tested in southern Florida (Jones et al., 1984).

Calculated values for PET differed considerably using the 2 methods. The Stephens-Stewart method gave values for PET much greater than those calculated using the Thornthwaite method. Water budgets calculated using PET values determined by the Stephens-Stewart method demonstrated frequent and prolonged water deficits for most all of the weather stations for which 30-year data was used. This is in contrast to data presented by Jones et al., (1984) and data generated using the Thornthwaite method. Using the Thornthwaite method to estimate PET, water deficit never occurred when water budgets were calculated using 30-year averages. When weather data for 1992 and 1993 (the time frame included in this study) was used for the calculation of PET and water budgets, again great differences were seen. Water budget data was visually compared to soil moisture conditions observed at snail habitat sites, and neither method was consistent in accurately predicting observed moisture conditions. At some ranch visits when pastures seemed quite dry, snail habitats were very wet.

In this study observable differences in soil moisture were often recorded from different sites on the same ranch. Major differences were also frequently recorded between the 3 ranches in southern Florida all of which were in fairly close proximity. If surface soil moisture had a simple relationship with available soil moisture as calculated from water budgets,

one would expect little variation between snail habitat sites in the same proximity. This was not the case. Similar findings were reported by Smith & Wilson (1980) in a study of variations in the microclimate of *Lymnaea truncatula* habitats. They concluded that: (1) standard meteorological measurements provide misleading indices of the microclimate of *Lymnaea truncatula* habitats; and (2) the habitats themselves are sufficiently diverse in form that the relationship between the prevailing weather conditions and the microclimate experienced by the snails and flukes depends upon features that are unique to that habitat. Data from the present study are concordant with these findings. Also, in many cases ranch to ranch variability in *F. cubensis* population dynamics appeared to be greater than regional variability between northern, central and southern Florida.

In this project, the seasonal transmission dynamics of *F. hepatica* was determined by studying the bionomics and infection prevalence of *F. cubensis* populations. This approach enabled multiple sites to be studied at a relatively low cost. The high cost of tracer animals would have prevented this study from being performed had that approach been necessary. The results of this study clearly indicate the usefulness of this alternate technique for the determination of seasonal transmission dynamics for *F. hepatica*. These results also suggest that care must be taken in the selection of pastures to include in a study such as

this. Although 7 ranches were included, only one (Brighton Seminole Indian Reservation) had both consistent snail populations and large numbers of infected snails over the course of the study. However, the data from this one ranch offers a fairly clear picture of the seasonal transmission patterns that exist for south-central Florida under fairly optimal conditions for fluke transmission.

Cattle on this ranch are treated once annually in March with flukicide (albendazole). The spring of 1992 was fairly dry and very few *Fossaria* were found during visits prior to the start of the study. However, on 4-24-92, at the start of the study, snail habitats were fairly wet and large numbers of *Fossaria* were found. Following very heavy June rainfall the habitat became essentially submerged and for the remainder of the summer only very small numbers of *Fossaria* were found. This site was fairly unique in this study as it was only 1 of 2 sites where *Fossaria* were found throughout the summer. The other site where *Fossaria* were found during the summer was at the DRU (original site 2) in northern Florida. However, this habitat was probably not a good representative location and it was dropped from the study (see results).

On 7-16-92 herd prevalence was 32%. Since cattle were treated with albendazole 3 months earlier, the flukes shedding eggs in these cattle were acquired after the 1st of January (immature and not susceptible to flukicide in March) and before May (flukes acquired after May 1st would not be

producing eggs by 7-16-92). Infection prevalence increased to 64% on 12-18-92. Flukes that would be producing eggs on this date would have been acquired prior to October. The increase in herd prevalence from July to December therefore is largely due to infections acquired between May and September. This suggests that summer transmission can occur. It is unknown, however, whether these infections were acquired predominantly in May or throughout the summer.

On 5-12-93, the herd infection prevalence (n=20) was 15% with a mean egg count per 2g of feces of 0.4. This low level of infection was a result of treatment given several weeks earlier. However, albendazole only kills mature flukes with high efficacy, therefore, flukes acquired after January would not be killed. Evidence of this was seen on 16 November 1993 when infection prevalence (n=20) was 100% and mean eggs per 2g of feces was 15.4. Since no snails were present on pasture between July and October, and hot dry summer weather would have rapidly killed remaining metacercariae, the flukes infecting the cattle in November were acquired the previous winter through early summer.

Snails were assayed for infection beginning in May 1993. On 5-12-93, 1 infected snail was detected. Soon after, the habitat dryed and snails entered into aestivation. Snails emerged from aestivation in early to middle November and on 16 November no infected snails were found. It would be expected that few of these survivors of the spring generation of snails

would be infected since cattle were shedding few eggs at that time. On the next collection (12-7-93), 7 infected snails were collected (point prevalence = 5.3%) indicating that the large number of eggs being shed by the cattle in the autumn quickly began to infect the snails. Infected snails were found continuously through the remainder of the study (4-26-94) with infection prevalences ranging from 2.2 to 5.2% per collection.

Following miracidial penetration it takes approximately 6 weeks for cercariae to fully develop within infected snails under Florida conditions. Both 1992 and 1993, at site 1 on the Brighton Seminole Indian Reservation, snails reemerged from aestivation and rapidly began reproducing in early November. Therefore, shedding of fluke eggs onto pasture in the autumn resulted in metacercarial contamination of pastures and subsequent fluke infection of cattle beginning around the 1st of January in both years. The end to fluke transmission each spring-summer will depend largely on spring rainfall. If snail habitats remain wet during the late spring and early summer, snails will remain on pasture further into the summer months before entering into aestivation. Metacercariae will then be shed for longer periods and the wet environment will enhance their survival. Hot dry weather during the late spring will cause snails to enter into aestivation earlier and metacercariae are rapidly killed under hot and dry conditions. The former situation occurred in 1992 and the latter in 1993.

It is interesting that in both years snail populations had similar dynamics despite rather large differences in summer rainfall. The only major difference was that in the wet summer of 1992, small numbers of snails were present, whereas in the dry summer of 1993 no snails were present.

The patterns seen on the Brighton Seminole Indian Reservation were also seen at the other research sites throughout the state with only minor variation. In 1992, *Fossaria* returned to pasture in northern Florida by late September and in central Florida by mid-October. This was approximately 3 and 6 weeks respectively earlier than in southern Florida. While this difference may be due to chance, it is likely that *Fossaria* reemerge from aestivation slightly earlier with lower autumn temperatures of northern Florida. In a 3-year study of *F. hepatica* transmission dynamics in northern Florida, transmission began in November (1 year), in December (1 year), and January (1 year) (Boyce & Courtney, 1990). Therefore, it is likely that transmission of *F. hepatica* begins 4 to 6 weeks earlier in northern Florida than southern Florida in some years. This pattern was not seen in 1993 but this was probably due to the extremely dry conditions that existed during the summer of 1993. At snail habitats that remained dry for a long period, i.e. from May to around October, *Fossaria* did not return to pasture in large numbers until well into the spring of 1994 (Wolfe Ranch, DRU, Creek Ranch). This snail generation was then forced into

aestivation by dry weather in April 1994. On these ranches, therefore, snails were not present long enough for *F. hepatica* to complete its intramoluscan development. On these ranches it is probable that no liver fluke transmission occurred during the autumn of 1993 or the winter and spring of 1994.

The time point when transmission ends each year depends largely on the amount of rainfall in April and May. Everywhere in the state of Florida, April and May are the driest months of the year, i.e., the difference between PET and average rainfall is the greatest. Because of this, nearly every year in the middle to late spring there is a short drought that is sufficient to cause snail habitats to dry and force snails to aestivate. When summer rains begin and replenish soil moisture, snails remain in aestivation until the autumn. If snail habitats do not dry during this time, snails may remain on pasture throughout the summer as was seen at Brighton Seminole Indian Reservation site 1 and DRU site 2 in 1992. Since eggs of *F. hepatica* can survive summer temperatures in Florida (see chapter 2), summer transmission will occur. In this 2-year study, sites in northern and central Florida dried earlier in the spring than did those in southern Florida. While this pattern could be due to chance, it is likely that transmission not only begins a little later in the autumn in southern Florida, but also continues for a longer period into the early summer.

Snail reproduction occurred during most of the year when snails were present on pasture. However, 2 distinct peaks of reproductive activity were noted in the early autumn and again in the spring (Figure 5-32). The autumn peak was a result of heavy egg laying by snails that survived the summer by aestivating. The majority of these spring generation snails lived for only 3 to 6 weeks after returning to the pasture surface but seeded habitats with large numbers of eggs that formed the new generation of snails. A second peak of reproductive activity was noted in the spring. In late spring, hot and dry weather causes snail habitats to desiccate and snail populations tend to suffer high rates of mortality. It seems likely therefore, that this spring peak in reproduction is an adaptation to ensure survival through the summer.

During periods of dry weather, water sources are sometimes augmented by continuously flowing artesian wells on cattle ranches in Florida. Batte & Swanson, (1951) reported that runoff from artesian wells may modify snail habitat to the extent that snails may remain active during periods of drought. Because the presence of continuously flowing wells could potentially alter seasonal transmission patterns, an additional ranch with artesian well-fed *F. cubensis* habitats (Rio Ranch) was also included in this project. Data from this ranch were very interesting because the seasonal dynamics of *F. cubensis* populations were very similar to other ranches in

the study. Few to no Fossaria were found during the summer at this ranch even though the habitats never dryed in the spring. It appeared that ecological changes that occurred in the habitat as a result of the tropical summer weather, caused the environment to become unfavorable to snails. This is best explained in terms of the ecological niche that *F. cubensis* exploits.

Fossaria cubensis are aquatic/amphibious snails that prefer a wet, muddy amphibious environment. Large populations of *F. cubensis* are almost never found in areas covered with vegetation or areas that are permanently flooded. Although I frequently observed *Fossaria* below the water surface during the cooler portions of the year, when surface soil and water temperatures were warm, *Fossaria* were almost always on the mud surface (if open mud surface was present) or attached to vegetation above the water line (if the area was flooded). During the early summer in Florida, tropical grasses and weeds grow at extraordinary rates. At every *Fossaria* habitat that remained wet during the summer (all sites in 1992, Rio Ranch in 1992 and 1993), the ecology of the habitat area changed such that virtually no amphibious niche was present. The entire area was either covered with vegetation or standing water. Under such conditions, large populations of *Fossaria* were never found and most often none or occasionally very few individual snails were present. This phenomena explains why the computer model of Malone et al. (1987) incorrectly

predicted that the summer months in southern Florida would be an important period of liver fluke transmission (Malone & Craig, 1990). Irrespective of spring drought, these ecological changes preclude the existence of large populations of *Fossaria* during summers in Florida. This factor combined with usual spring drought probably results in little to no summer transmission occurring in most years. It is important to note, however, that the flooded pasture conditions that are frequently present in the summers provide optimal opportunity for snail infection and cercarial dispersion. Therefore, even a small number of snails could potentially cause an important level of liver fluke transmission in some years. This would most likely be in years with wet springs followed by summers with normal or above normal rainfall.

Most of the inferences regarding liver fluke transmission made in this report are based on the seasonal dynamics of *F. cubensis* populations. While much is made of the importance of accurate snail intermediate host infection prevalence data, on only 1 ranch did the data yield important epidemiological information. At first glance then, the results of the snail intermediate host infection prevalence data reported here seem to indicate a failure of this method to accurately predict the likely transmission patterns in Florida on all but 1 ranch. To the contrary, however, these results show the potential bias that can be introduced when only 1 or 2 sites are used in a study. Seasonal transmission dynamics of *F. hepatica* on any

single location are influenced by numerous factors, some of which can differ greatly from ranch to ranch and from year to year. For a given locale, geophysical factors such as climate, soil type and topography are fairly stable, however yearly precipitation patterns, management practices, and the physiological status of the animals on pasture can vary greatly. Depending upon the inputs of these factors, any single location may not be representative of the region and any single year may not representative of an average year in terms of liver fliuke transmission. On those ranches where very few infected snails were found, tracer animals would likewise have acquired very few flukes. If tracer calf data was the only data available, then the paucity of flukes in these animals would leave the researcher with no useful information on transmission dynamics. This situation occurred in a tracer calf study performed in the Texas Gulf Coast (Craig & Bell, 1978). Three calves were placed on pasture at monthly intervals for 1 year at 2 separate sites. At one of these sites only 1 fluke was acquired the entire year out of 35 calves that were used. The only thing that rescued this study was that a large number of *Fascioloides magna* were acquired by the calves. Since these flukes use the same snail intermediate host as *F. hepatica*, it was assumed that transmission patterns for both flukes would be similar.

All ranches used in this study had a history of liver fluke infection and were privately owned (except DRU) with

individualized management systems. The ideal situation for generating useful data in a study such as this would be where high levels of transmission are occurring. However, these ranches were only willing to cooperate in data collection and were not willing to change management schemes to promote liver fluke transmission. Differences in snail infection prevalences seen between the ranches were due largely to management differences that affected both the overall herd prevalence and the amount of time cattle spent on pastures where snails were collected. The most important of these management differences seemed to be the past and current treatment program for liver flukes, stocking rate and pasture utilization. The two ranches where cattle were permanently pastured and treatment was irregular or poorly timed (Seminole and Williams) had the highest herd prevalence and the highest snail infection prevalences. On the other ranches, cattle had much lower levels of infection and were frequently removed from pastures where snails were collected for extended periods of time as part of a pasture rotation program designed to optimize forage utilization. Absence of cattle on these pastures also reduced the size of snail habitats by minimizing the amount of trampling activity in and around snail habitats. Herd prevalence was not determined at regular intervals for these ranches because cattle frequently were not present, however, when checked, prevalences ranged from 0% (0 eggs per 2g of feces) to 50% (1.06 eggs per 2g of feces).

The overall goal of this project was to determine the seasonal transmission dynamics for *F. hepatica* in the different climatic regions of Florida. Although only 2 years of data were collected, the broad range of research sites included and the differences in precipitation that occurred between the 2 years lends support to the conclusions presented here. Another goal of this project was to develop a new, superior method for the detection of *F. hepatica* infected snails. This was also accomplished. Since there is a limit to the amount of data that can be collected in a single dissertation project, this work can best be viewed as a pilot research project. I have shown that by combining snail intermediate host bionomics and infection prevalence data, the seasonal transmission dynamics of *F. hepatica* can be determined. Since there is apparently little difference in the transmission dynamics between northern and southern Florida, future research could modify the design used here to more accurately study a few sites for 3 to 5 years. It would also be useful to combine this work with a tracer animal study. This would lead to a better understanding of the relationship between snail population dynamics, snail infection prevalence and fluke acquisition by grazing animals. Data on this relationship could then be used to predict fluke acquisition without the great expense of tracer animals in future studies elsewhere.

It is clear that during most of the year (September to June) that available soil moisture is the most important factor regulating *F. cubensis* populations. Future research should address this issue by collecting better data on the microclimate of snail habitats and correlating it to available meteorological data. It should be possible with several years of precise data to develop a simple model to predict relative fluke risk for individual years. This model could also include snail infection prevalence data to customize the output to individual ranches. This is important because relative risk will depend not only on climatic factors but also on management factors that could greatly effect the herd infection prevalence.

Finally, it is important to use the knowledge gained in this project to make improved control recommendations for Florida cattlemen. Previous recommendations for a single annual treatment in early autumn on all properties where liver flukes are enzootic (Courtney et al., 1985, Shearer et al., 1986) are essentially unchanged. However, the timing of this treatment should be modified. To obtain the maximum benefit of autumn treatment, it must be given prior to the reemergence of snails. In chapter 2 it was demonstrated that eggs of *F. hepatica* can survive in fecal pats for at least 5 weeks during the summer. Under natural conditions, fecal pats deposited in areas surrounding wet snail habitats will disintegrate within a few weeks. Therefore, treatment should be given at least 4

weeks before snails repopulate habitat areas. This will ensure that eggs in snail habitat areas have sufficient time to develop and hatch at a time when miracidia do not have an opportunity to infect snails. A problem in making broad recommendations is that snails will repopulate pastures at slightly different times each autumn depending upon interactions of climatic and environmental variables that differ each year. Since snails can repopulate pastures as early as late September in northern Florida, treatment should be given before Labor Day, preferably during late August. An exception to this would be in years with below normal rainfall. If snail habitats remain dry in the late summer and early autumn, treatment could be postponed for 1 month. In southern Florida, treatment should be given before October 1st.

The question of whether or not to give cattle an additional spring treatment remains unresolved. Previous recommendations were to give a spring treatment in unusually wet years or on properties with a history of severe fascioliasis. Without data on the cost-benefit of spring treatment, these recommendations remain unchanged. The problem with spring treatment is that flukicidal drugs do not kill juvenile flukes. Therefore, a large percentage of the flukes that are infecting cattle in the spring will not be killed, even with a full dose of clorsulon. Even so, if cows harbor large fluke burdens, they and their calves will most

likely benefit from treatment. Calves infected with liver flukes will have impaired weight gains for up to 6 months, but the majority of losses occur during the first 4 months after infection (Hope Cawdery et al., 1977). Calves will thus benefit from treatment in the spring if the timing is correct. Because the age of calves and grazing management are extremely important in determining the proper timing of treatment and whether or not treatment is warranted, broad recommendations cannot be made. This decision should be made by ranchers after consulting with their veterinarian. However, it may be advisable to treat all replacement heifers in the spring. This is because (1) these animals are young enough that they are still highly susceptable to infection, (2) the fertility and productivity of this group is extremely important to the economic future of the herd, and (3) replacement heifers often receive less attention to matters of health and nutrition than do other groups of animals. Choice of drugs is important when treating in the spring and a full dose of clorsulon should be used.

Properly timed treatment with flukicidal drugs is the cornerstone of liver fluke control and will remain so for the foreseeable future. However, creative pasture management has the potential to play an important role in an overall control program. The logic used in the "dose and move" control program for gastrointestinal nematodes (Michel, 1969) can also be used for liver fluke control. Most cattle ranches in

Florida are large enough that some pastures will harbor populations of *F. cubensis* and some will not, due to differences in pasture physiographics. Advantage can be taken of this situation to minimize fluke transmission. The following is one but not the only possible pasture management scheme that can be used.

Cattle are moved from pastures with large populations of *F. cubensis* in January and kept on "snail free" pastures during January, February and March. This will allow snails to become infected in the late autumn and early winter but will prevent cows from becoming infected since they are removed from pasture before cercariae are produced. By April, most infected snails will have died and a large percentage of metacercariae shed onto pasture in January and February will have died. Some fluke transmission will occur from residual metacercariae but any new snails that become infected will die or aestivate before shedding cercariae. Since the poorest quality pasture of the year is in January, February and March, cattle are usually heavily supplemented during this time. Heavier stocking rates will therefore not have the same adverse effect on nutrition that it would during other times of the year. This pasture management scheme combined with annual late summer/early autumn treatment could potentially reduce levels of liver fluke transmission below an economic threshold. Even if it is not possible to keep cows off of "fluky" pastures for 3 months, any time the cows are kept off

of such pastures between January and May will be beneficial in reducing fluke burdens.

It is unlikely that liver flukes can be erradicated from a property once established in the herd. However, twice yearly treatment for several years may reduce transmission levels below an economic threshold. Low-level transmission could then be maintained with only a single treatment given once each year in the late summer. Combining this treatment scheme with sound pasture management would probably eliminate or at least greatly minimize production losses resulting from infection with liver flukes. Once liver fluke transmission has been reduced to a low level, late summer treatment might even be skipped without adverse consequences in years with subnormal rainfall.

Table 5-1. *Fasciola hepatica* and *Paramphistomum* spp. egg count data. 1992 data are from 25 fecal samples per date and data from 1993 and 1994 are from 20 fecal samples.

		Fasciola hepatica			*Paramphistomum* spp.		
Ranch	Date	Prevalence	Avg EPG†	ESI*	Prevalence	Avg EPG†	ESI*
Wolfe	08/03/92	84.0	2.3	3.9	68.0	1.9	2.6
Wolfe	12/18/92	48.0	2.1	2.0	76.0	4.4	6.7
Wolfe	06/02/93	40.0	17.5	14.0	85.0	6.2	10.5
Wolfe	04/27/94	0.0	0.0	0.0	90.0	2.5	4.5
	Avg	43.0	5.5	5.0	79.8	3.7	6.1
Osceola	07/16/92	32.0	0.4	0.3	88.0	7.0	12.4
Osceola	12/18/92	64.0	1.3	1.6	100.0	24.0	48.1
Osceola	05/12/93	15.0	0.2	0.1	90.0	21.5	38.7
Osceola	11/16/93	100.0	7.7	15.4	100.0	16.8	33.6
Osceola	04/26/94	15.0	0.2	0.1	90.0	10.1	18.2
	Avg	45.2	2.0	3.5	93.6	15.9	30.2
Williams	05/12/93	63.0	4.3	5.5	100.0	7.2	14.4
Williams	11/16/93	20.0	0.1	0.04	90.0	27.4	49.4
Williams	04/26/94	5.0	0.4	0.04	90.0	4.3	7.8
	Avg	29.3	1.6	1.9	93.3	13.0	23.9
Rio	08/13/92	0.0	0.0	0.0	73.0	2.3	3.4
Creek	02/08/94	5.0	0.03	0.003	75.0	2.0	3.0
Deseret	04/05/94	50.0	0.6	0.6	55.0	1.0	1.0
	Total Avg	36.1	2.5	2.9	84.7	9.2	17.0
	Max	100.0	17.5	15.4	100.0	27.4	49.4
	Min	0.0	0.0	0.0	55.0	1.0	1.0
	Std	29.5	4.4	4.7	12.0	8.3	15.9

† EPG = Eggs Per Gram of Feces

* ESI = Egg Shedding Index: (Average EP2G x Herd Prevalence)/100

Table 5-2. Gainesville 2 WSW (30-year) and Gainesville Municipal Airport (1992-1994): Water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.2	3.5	4.0	3.8	2.0	12.9	1.5	8.6	4.4	5.7	2.1	0.7	54.3
Potential Evapotranspiration (PET)	2.5	3.1	3.9	4.9	6.1	7.4	7.7	6.8	5.8	3.9	3.0	2.1	57.1
P - PET	2.7	0.3	0.1	-1.2	-4.1	5.5	-6.1	1.8	-1.4	1.8	-0.9	-1.3	-2.8
Change in Stored Soil Moisture (Δ ST)	2.7	0.3	0.1	-1.2	-2.9	5.5	-5.5	1.8	-1.4	1.8	-0.9	-1.3	-1.0
Total Available Soil Moisture (ST)	3.7	4.0	4.1	2.9	0.0	5.5	0.0	1.8	0.4	2.2	1.3	0.0	25.9
Actual Evapotranspiration (AE)	2.5	3.1	3.9	4.9	4.9	7.4	7.0	6.8	5.8	3.9	3.0	2.1	55.3
Deficit (D)	0.0	0.0	0.0	0.0	1.2	0.0	0.7	0.0	0.0	0.0	0.0	0.0	1.9
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	3.3	4.8	4.6	0.9	1.4	6.1	3.4	5.7	2.0	8.0	1.4	2.2	43.7
Potential Evapotranspiration (PET)	3.1	2.6	3.8	4.8	6.2	7.4	7.7	7.1	5.9	4.2	2.8	1.8	57.4
P - PET	0.1	2.2	0.8	-3.9	-4.8	-1.3	-4.2	-1.5	-3.9	3.8	-1.5	0.4	-13.8
Change in Stored Soil Moisture (Δ ST)	0.1	2.2	0.8	-3.1	0.0	0.0	0.0	0.0	0.0	3.8	-1.5	0.4	2.7
Total Available Soil Moisture (ST)	0.1	2.3	3.1	0.0	0.0	0.0	0.0	0.0	0.0	3.8	2.3	2.7	14.3
Actual Evapotranspiration (AE)	3.1	2.6	3.8	4.0	1.4	6.1	3.4	5.7	2.0	4.2	2.8	1.8	40.9
Deficit (D)	0.0	0.0	0.0	0.8	4.8	1.3	4.3	1.4	3.9	0.0	0.0	0.0	16.5
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1994	JAN	FEB	TOTAL
Precipitation (P)	9.0	0.4	9.4
Potential Evapotranspiration (PET)	2.5	3.2	5.7
P - PET	6.5	-2.8	3.7
Change in Stored Soil Moisture (Δ ST)	3.3	-2.8	0.5
Total Available Soil Moisture (ST)	6.0	3.2	9.2
Actual Evapotranspiration (AE)	2.5	3.2	5.7
Deficit (D)	0.0	0.0	0.0
Surplus (S)	3.2	0.0	3.2

30 Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	3.3	3.9	3.7	2.9	4.2	6.6	7.1	8.0	5.6	2.3	2.0	3.2	52.8
Potential Evapotranspiration (PET)	2.8	3.0	4.2	5.5	6.7	7.5	7.6	7.0	6.0	4.3	2.9	2.2	59.6
P - PET	0.4	0.9	-0.5	-2.5	-2.5	-0.8	-0.5	1.0	-0.4	-2.0	-0.9	1.0	-6.8
Change in Stored Soil Moisture (Δ ST)	0.4	0.9	-0.5	-1.8	0.0	0.0	0.0	1.0	-0.4	-0.6	0.0	1.0	0.0
Total Available Soil Moisture (ST)	1.4	2.3	1.8	0.0	0.0	0.0	0.0	1.0	0.6	0.0	0.0	1.0	8.1
Actual Evapotranspiration (AE)	2.8	3.0	4.2	4.8	4.2	6.6	7.1	7.0	6.0	2.9	2.0	2.2	52.8
Deficit (D)	0.0	0.0	0.0	0.7	2.5	0.8	0.5	0.0	0.0	1.4	0.9	0.0	6.8
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 5-3. Gainesville 2 WSW (30-year) and Gainesville Municipal Airport (1992-1994): Water budgets calculated using a 6 inch model for soil moisture storage and using the Thornthwaite method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.2	3.5	4.0	3.8	2.0	12.9	1.5	8.6	4.4	5.7	2.1	0.7	54.3
Potential Evapotranspiration (PET)	0.8	1.3	1.9	2.6	4.2	6.0	7.3	6.1	5.1	2.7	2.0	1.1	41.0
P - PET	4.4	2.2	2.1	1.2	-2.2	6.8	-5.8	2.5	-0.7	3.0	0.1	-0.3	13.3
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	0.0	-2.2	2.2	-5.8	2.5	-0.7	3.0	0.1	-0.3	-1.2
Total Available Soil Moisture (ST)	6.0	6.0	6.0	6.0	3.8	6.0	0.2	2.7	2.0	5.0	5.1	4.8	53.6
Actual Evapotranspiration (AE)	0.8	1.3	1.9	2.6	4.2	6.0	7.3	6.1	5.1	2.7	2.0	1.1	41.1
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	4.4	2.2	2.1	1.2	0.0	4.6	0.0	0.0	0.0	0.0	0.0	0.0	14.5

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	3.3	4.8	4.6	0.9	1.4	6.1	3.4	5.7	2.0	8.0	1.4	2.2	43.7
Potential Evapotranspiration (PET)	1.5	0.8	1.8	2.4	4.4	6.0	7.3	7.0	5.3	3.3	1.7	0.7	42.2
P - PET	1.7	4.0	2.9	-1.5	-3.0	0.0	-3.9	-1.3	-3.3	4.7	-0.4	1.5	1.5
Change in Stored Soil Moisture (Δ ST)	1.2	0.0	0.0	-1.5	-3.0	0.0	-1.5	0.0	0.0	4.7	-0.4	1.5	1.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	4.5	1.5	1.5	0.0	0.0	0.0	4.7	4.3	5.8	40.3
Actual Evapotranspiration (AE)	1.5	0.8	1.8	2.4	4.4	6.0	4.9	5.7	2.0	3.3	1.7	0.7	35.2
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	2.4	1.3	3.3	0.0	0.0	0.0	7.0
Surplus (S)	0.5	4.0	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.4

1994	JAN	FEB	TOTAL
Precipitation (P)	9.0	0.4	9.4
Potential Evapotranspiration (PET)	0.8	1.5	2.3
P - PET	8.2	-1.0	7.2
Change in Stored Soil Moisture (Δ ST)	0.2	-1.0	-0.8
Total Available Soil Moisture (ST)	6.0	5.0	11.0
Actual Evapotranspiration (AE)	0.8	1.5	2.3
Deficit (D)	0.0	0.0	0.0
Surplus (S)	8.0	0.0	8.0

30 Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	3.3	3.9	3.7	2.9	4.2	6.6	7.1	8.0	5.6	2.3	2.0	3.2	52.8
Potential Evapotranspiration (PET)	1.1	1.1	2.3	3.4	5.3	6.2	7.1	6.7	5.4	3.6	1.9	1.3	45.5
P - PET	2.1	2.8	1.3	-0.5	-1.1	0.4	-0.0	1.3	0.2	-1.2	0.1	1.9	7.4
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	-0.5	-1.1	0.4	0.0	1.2	0.0	-1.2	0.1	1.1	0.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	5.5	4.4	4.8	4.8	6.0	6.0	4.8	4.9	6.0	65.2
Actual Evapotranspiration (AE)	1.1	1.1	2.3	3.4	5.3	6.2	7.1	6.7	5.4	3.6	1.9	1.3	45.4
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	2.1	2.8	1.3	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.8	7.3

Table 5-4. St. Augustine WFOY: Water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	3.9	1.6	3.6	0.2	4.5	9.2	3.2	4.5	13.6	4.6	2.1	0.4	51.4
Potential Evapotranspiration (PET)	2.3	3.2	4.1	5.1	6.3	7.6	7.9	7.0	5.9	4.2	3.1	2.3	59.1
P - PET	1.6	-1.6	-0.5	-4.8	-1.9	1.5	-4.8	-2.5	7.7	0.4	-1.0	-1.9	-7.7
Change in Stored Soil Moisture (Δ ST)	1.6	-1.6	-0.5	-0.5	0.0	1.5	-1.5	0.0	6.0	0.0	-1.0	-1.9	2.1
Total Available Soil Moisture (ST)	2.6	1.0	0.5	0.0	0.0	1.5	0.0	0.0	6.0	6.0	5.0	3.1	25.7
Actual Evapotranspiration (AE)	2.3	3.2	4.1	0.7	4.5	7.6	4.7	4.5	5.9	4.2	3.1	2.3	47.1
Deficit (D)	0.0	0.0	0.0	4.4	1.9	0.0	3.2	2.5	0.0	0.0	0.0	0.0	12.0
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.4	0.0	0.0	2.1

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	4.6	3.4	4.6	0.3	2.5	0.6	2.4	3.4	6.1	11.8	2.4	2.3	44.5
Potential Evapotranspiration (PET)	3.3	2.8	3.9	5.1	6.3	7.6	7.8	7.3	6.0	4.4	3.0	1.9	59.3
P - PET	1.3	0.6	0.7	-4.8	-3.8	-7.0	-5.4	-3.8	0.1	7.4	-0.6	0.4	-14.8
Change in Stored Soil Moisture (Δ ST)	1.3	0.6	0.7	-4.8	-0.9	0.0	0.0	0.0	0.1	5.9	-0.6	0.4	2.7
Total Available Soil Moisture (ST)	4.4	5.0	5.7	0.9	0.0	0.0	0.0	0.0	0.1	6.0	5.4	5.8	33.3
Actual Evapotranspiration (AE)	3.3	2.8	3.9	5.1	3.4	0.6	2.4	3.4	6.0	4.4	3.0	1.9	40.2
Deficit (D)	0.0	0.0	0.0	0.0	2.9	7.0	5.4	3.8	0.0	0.0	0.0	0.0	19.1
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0	1.5

1994	JAN	FEB	TOTAL
Precipitation (P)	6.9	1.9	8.9
Potential Evapotranspiration (PET)	2.6	3.2	5.8
P - PET	4.3	-1.3	3.0
Change in Stored Soil Moisture (Δ ST)	0.2	-1.3	-1.1
Total Available Soil Moisture (ST)	6.0	4.7	10.7
Actual Evapotranspiration (AE)	2.6	3.2	5.8
Deficit (D)	0.0	0.0	0.0
Surplus (S)	4.1	0.0	4.1

Table 5-5. St. Augustine WFOY: Water budgets calculated using a 6 inch model for soil moisture storage and using the Thornthwaite method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	3.9	1.6	3.6	0.2	4.5	9.2	3.2	4.5	13.6	4.6	2.1	0.4	51.4
Potential Evapotranspiration (PET)	1.0	1.3	2.0	2.6	4.5	6.6	8.1	6.7	5.3	3.1	2.1	1.4	44.7
P - PET	2.9	0.4	1.6	-2.3	-0.1	2.5	-4.9	-2.2	8.3	1.5	-0.0	-1.0	6.7
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	-2.3	-0.1	2.4	-4.9	-1.1	6.0	0.0	0.0	-1.0	-1.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	3.7	3.6	6.0	1.1	0.0	6.0	6.0	6.0	5.0	55.4
Actual Evapotranspiration (AE)	1.0	1.3	2.0	2.6	4.5	6.6	8.1	5.6	5.3	3.1	2.1	1.4	43.6
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	1.1
Surplus (S)	2.9	0.4	1.6	0.0	0.0	0.1	0.0	0.0	2.3	1.5	0.0	0.0	8.8

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	4.6	3.4	4.6	0.3	2.5	0.6	2.4	3.4	6.1	11.8	2.4	2.3	44.5
Potential Evapotranspiration (PET)	1.6	0.8	1.7	2.6	4.5	6.6	7.8	7.4	5.6	3.5	1.8	0.8	44.8
P - PET	3.0	2.6	2.8	-2.3	-2.0	-6.0	-5.3	-3.9	0.5	8.2	0.6	1.6	-0.3
Change in Stored Soil Moisture (Δ ST)	1.0	0.0	0.0	-2.3	-2.0	-1.7	0.0	0.0	0.5	5.5	0.0	0.0	1.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	3.7	1.7	0.0	0.0	0.0	0.5	6.0	6.0	6.0	41.9
Actual Evapotranspiration (AE)	1.6	0.8	1.7	2.6	4.5	2.3	2.4	3.4	5.6	3.5	1.8	0.8	31.0
Deficit (D)	0.0	0.0	0.0	0.0	0.0	4.3	5.3	3.9	0.0	0.0	0.0	0.0	13.5
Surplus (S)	2.0	2.6	2.8	0.0	0.0	0.0	0.0	0.0	0.0	2.7	0.6	1.6	12.3

1994	JAN	FEB	TOTAL
Precipitation (P)	6.9	1.9	8.9
Potential Evapotranspiration (PET)	0.8	1.3	2.1
P - PET	6.2	0.6	6.8
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0
Total Available Soil Moisture (ST)	6.0	6.0	12.0
Actual Evapotranspiration (AE)	0.8	1.3	2.1
Deficit (D)	0.0	0.0	0.0
Surplus (S)	6.2	0.6	6.8

Table 5-6. Melbourne WSO: Water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	1.4	3.3	4.0	4.2	1.5	12.3	2.9	5.8	7.2	2.7	2.6	1.5	49.4
Potential Evapotranspiration (PET)	2.6	3.8	4.5	5.4	6.3	7.6	7.6	7.0	6.1	4.7	3.6	2.7	61.9
P - PET	-1.2	-0.5	-0.5	-1.2	-4.9	4.7	-4.7	-1.2	1.1	-2.0	-1.0	-1.2	-12.6
Change in Stored Soil Moisture (Δ ST)	-0.3	0.0	0.0	0.0	0.0	4.7	-4.7	0.0	1.1	-1.1	0.0	0.0	-0.3
Total Available Soil Moisture (ST)	0.0	0.0	0.0	0.0	0.0	4.7	0.0	0.0	1.1	0.0	0.0	0.0	5.8
Actual Evapotranspiration (AE)	1.7	3.3	4.0	4.2	1.5	7.6	7.6	5.8	6.1	3.8	2.6	1.5	49.7
Deficit (D)	0.9	0.5	0.5	1.2	4.9	0.0	0.0	1.2	0.0	0.9	1.0	1.2	12.3
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.2	1.8	8.6	1.8	2.0	1.3	4.0	3.0	5.4	4.6	1.2	0.5	39.3
Potential Evapotranspiration (PET)	3.2	3.3	4.4	5.2	6.6	7.5	7.6	7.1	6.1	4.9	3.5	2.4	61.7
P - PET	2.1	-1.5	4.2	-3.4	-4.6	-6.2	-3.6	-4.1	-0.7	-0.2	-2.3	-1.9	-22.4
Change in Stored Soil Moisture (Δ ST)	2.1	-1.5	4.2	-3.4	-1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Available Soil Moisture (ST)	2.1	0.6	4.8	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.9
Actual Evapotranspiration (AE)	3.2	3.3	4.4	5.2	3.4	1.3	4.0	3.0	5.4	4.6	1.2	0.5	39.5
Deficit (D)	0.0	0.0	0.0	0.0	3.2	6.2	3.6	4.1	0.7	0.3	2.3	1.9	22.3
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1994	JAN	FEB	TOTAL
Precipitation (P)	3.2	3.3	6.5
Potential Evapotranspiration (PET)	2.8	3.9	6.7
P - PET	0.4	-0.6	-0.2
Change in Stored Soil Moisture (Δ ST)	0.4	-0.4	0.0
Total Available Soil Moisture (ST)	0.4	0.0	0.4
Actual Evapotranspiration (AE)	2.8	3.7	6.5
Deficit (D)	0.0	0.2	0.2
Surplus (S)	0.0	0.0	0.0

30 Year Data	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.3	2.9	2.9	2.2	4.1	6.2	5.6	4.9	7.6	5.0	2.7	1.9	48.2
Potential Evapotranspiration (PET)	2.8	3.5	4.6	5.7	6.7	7.4	7.5	7.1	6.1	4.7	3.4	2.8	62.1
P - PET	-0.5	-0.6	-1.6	-3.5	-2.6	-1.2	-1.9	-2.2	1.5	0.2	-0.7	-0.7	-13.9
Change in Stored Soil Moisture (Δ ST)	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.2	-0.7	-0.7	0.6
Total Available Soil Moisture (ST)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.7	1.0	0.3	4.5
Actual Evapotranspiration (AE)	2.6	2.9	2.9	2.2	4.1	6.2	5.6	4.9	6.1	4.7	3.4	2.6	48.2
Deficit (D)	0.2	0.6	1.6	3.5	2.6	1.2	1.9	2.2	0.0	0.0	0.0	0.0	13.8
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 5-7. Melbourne WSO: Water budgets calculated using a 6 inch model for soil moisture storage and using the Thornthwaite method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	1.4	3.3	4.0	4.2	1.5	12.3	2.9	5.8	7.2	2.7	2.6	1.5	49.4
Potential Evapotranspiration (PET)	1.1	1.7	2.3	3.0	4.4	6.6	7.4	6.8	5.6	3.9	2.8	1.7	47.4
P - PET	0.3	1.5	1.7	1.2	-2.9	5.7	-4.5	-0.9	1.6	-1.2	-0.3	-0.2	1.9
Change in Stored Soil Moisture (Δ ST)	0.3	1.5	1.6	0.0	-2.9	2.9	-4.5	-0.9	1.6	-1.2	-0.3	-0.2	-2.1
Total Available Soil Moisture (ST)	2.9	4.4	6.0	6.0	3.1	6.0	1.5	0.6	2.2	1.0	0.7	0.5	34.9
Actual Evapotranspiration (AE)	1.1	1.7	2.3	3.0	4.4	6.6	7.4	6.8	5.6	3.9	2.8	1.7	47.3
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.1	1.2	0.0	2.8	0.0	0.0	0.0	0.0	0.0	0.0	4.1

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.2	1.8	8.6	1.8	2.0	1.3	4.0	3.0	5.4	4.6	1.2	0.5	39.3
Potential Evapotranspiration (PET)	2.2	1.2	2.2	2.6	5.0	6.3	7.4	7.1	5.6	4.4	2.7	1.1	47.7
P - PET	3.0	0.6	6.4	-0.8	-3.0	-5.0	-3.5	-4.1	-0.2	0.3	-1.4	-0.6	-8.4
Change in Stored Soil Moisture (Δ ST)	3.0	0.6	1.9	-0.8	-3.0	-2.2	0.0	0.0	0.0	0.3	-0.3	0.0	-0.5
Total Available Soil Moisture (ST)	3.5	4.1	6.0	5.2	2.2	0.0	0.0	0.0	0.0	0.3	0.0	0.0	21.3
Actual Evapotranspiration (AE)	2.2	1.2	2.2	2.6	5.0	3.5	4.0	3.0	5.4	4.4	1.5	0.5	35.5
Deficit (D)	0.0	0.0	0.0	0.0	0.0	2.8	3.5	4.1	0.2	0.0	1.2	0.6	12.4
Surplus (S)	0.0	0.0	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5

1994	JAN	FEB	TOTAL
Precipitation (P)	3.2	3.3	6.5
Potential Evapotranspiration (PET)	1.6	2.1	3.6
P - PET	1.6	1.3	2.9
Change in Stored Soil Moisture (Δ ST)	1.6	1.3	2.9
Total Available Soil Moisture (ST)	1.6	2.9	4.5
Actual Evapotranspiration (AE)	1.6	2.1	3.7
Deficit (D)	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.0

30 Year Data	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.3	2.9	2.9	2.2	4.1	6.2	5.6	4.9	7.6	5.0	2.7	1.9	48.2
Potential Evapotranspiration (PET)	1.4	1.3	2.5	3.5	5.2	6.1	7.2	6.9	5.6	4.1	2.3	1.6	47.7
P - PET	0.8	1.5	0.4	-1.3	-1.1	0.1	-1.6	-2.0	2.0	0.9	0.3	0.3	0.4
Change in Stored Soil Moisture (Δ ST)	0.8	1.5	0.1	-1.3	-1.1	0.1	-1.6	-2.0	2.0	0.9	0.3	0.3	-0.0
Total Available Soil Moisture (ST)	4.4	5.9	6.0	4.7	3.6	3.7	2.1	0.1	2.1	3.0	3.3	2.6	41.5
Actual Evapotranspiration (AE)	1.4	1.3	2.5	3.5	5.2	6.1	7.2	6.9	5.6	4.1	2.3	1.6	47.7
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3

Table 5-8. Mountain Lake: Water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	1.3	3.8	0.8	4.6	3.0	9.1	3.2	11.8	6.3	3.2	3.5	0.8	51.4
Potential Evapotranspiration (PET)	2.7	3.8	4.6	5.6	6.5	7.6	7.7	6.9	6.2	4.5	3.6	2.7	62.4
P - PET	-1.4	0.0	-3.7	-1.0	-3.4	1.5	-4.5	4.9	0.1	-1.3	-0.1	-2.0	-11.0
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	0.0	0.0	1.5	-1.5	4.9	0.1	-1.3	-0.1	-2.0	1.6
Total Available Soil Moisture (ST)	0.0	0.0	0.0	0.0	0.0	1.5	0.0	4.9	5.0	3.7	3.6	1.6	20.3
Actual Evapotranspiration (AE)	1.3	3.8	0.8	4.6	3.0	7.6	4.7	6.9	6.2	4.5	3.6	2.7	49.7
Deficit (D)	1.4	0.0	3.7	1.0	3.4	0.0	3.0	0.0	0.0	0.0	0.0	0.0	12.5
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.8	3.1	4.6	4.2	2.4	3.3	5.9	3.8	6.8	2.0	0.3	1.0	43.0
Potential Evapotranspiration (PET)	3.2	3.3	4.4	5.3	6.6	7.6	7.6	7.1	6.1	4.8	3.4	2.4	61.8
P - PET	2.6	-0.2	0.2	-1.1	-4.2	-4.3	-1.8	-3.3	0.6	-2.8	-3.1	-1.4	-18.8
Change in Stored Soil Moisture (Δ ST)	2.6	-0.2	0.2	-1.1	-3.1	0.0	0.0	0.0	0.6	-0.6	0.0	0.0	-1.6
Total Available Soil Moisture (ST)	4.2	4.0	4.2	3.1	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	16.1
Actual Evapotranspiration (AE)	3.2	3.3	4.4	5.3	5.5	3.3	5.9	3.8	6.1	2.6	0.3	1.0	44.7
Deficit (D)	0.0	0.0	0.0	0.0	1.1	4.3	1.8	3.3	0.0	2.2	3.1	1.4	17.2
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1994	JAN	FEB	TOTAL
Precipitation (P)	4.2	2.3	6.5
Potential Evapotranspiration (PET)	2.8	3.9	6.7
P - PET	1.4	-1.6	-0.2
Change in Stored Soil Moisture (Δ ST)	1.4	-1.4	0.0
Total Available Soil Moisture (ST)	1.4	0.0	1.4
Actual Evapotranspiration (AE)	2.8	3.7	6.5
Deficit (D)	0.0	0.2	0.2
Surplus (S)	0.0	0.0	0.0

30 Year Data	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.5	3.1	3.3	2.2	4.6	7.2	7.8	8.0	6.1	2.7	2.0	2.0	51.3
Potential Evapotranspiration (PET)	2.7	3.5	4.6	5.8	6.9	7.5	7.6	7.1	6.2	4.7	3.3	2.6	62.6
P - PET	-0.2	-0.4	-1.3	-3.6	-2.3	-0.3	0.2	0.9	-0.1	-2.0	-1.4	-0.7	-11.2
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.9	-0.1	-1.0	0.0	0.0	0.0
Total Available Soil Moisture (ST)	0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.1	1.0	0.0	0.0	0.0	2.3
Actual Evapotranspiration (AE)	2.5	3.1	3.3	2.2	4.6	7.2	7.6	7.1	6.2	3.7	2.0	2.0	51.5
Deficit (D)	0.2	0.4	1.3	3.6	2.3	0.3	0.0	0.0	0.0	1.0	1.4	0.7	11.2
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 5-9. Mountain Lake: Water budgets calculated using a 6 inch model for soil moisture storage and using the Thornthwaite method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	1.3	3.8	0.8	4.6	3.0	9.1	3.2	11.8	6.3	3.2	3.5	0.8	51.4
Potential Evapotranspiration (PET)	1.2	1.7	2.5	3.1	4.6	6.6	7.8	6.4	5.9	3.4	2.8	1.7	47.9
P - PET	0.1	2.1	-1.7	1.4	-1.6	2.5	-4.6	5.4	0.4	-0.2	0.7	-1.0	3.5
Change in Stored Soil Moisture (Δ ST)	0.1	0.9	-1.7	1.4	-1.6	1.9	-4.6	4.6	0.0	-0.2	0.2	-1.0	-0.0
Total Available Soil Moisture (ST)	5.1	6.0	4.3	5.7	4.1	6.0	1.4	6.0	6.0	5.8	6.0	5.0	61.4
Actual Evapotranspiration (AE)	1.2	1.7	2.5	3.1	4.6	6.6	7.8	6.4	5.9	3.4	2.8	1.7	47.7
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.0	1.2	0.0	0.0	0.0	0.6	0.0	0.8	0.4	0.0	0.5	0.0	3.5

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.8	3.1	4.6	4.2	2.4	3.3	5.9	3.8	6.8	2.0	0.3	1.0	43.0
Potential Evapotranspiration (PET)	2.2	1.2	2.1	2.7	4.9	6.6	7.4	7.1	5.6	4.1	2.3	1.1	47.4
P - PET	3.6	1.9	2.4	1.5	-2.6	-3.3	-1.6	-3.3	1.2	-2.1	-2.0	-0.1	-4.4
Change in Stored Soil Moisture (Δ ST)	1.0	0.0	0.0	0.0	-2.6	-3.3	-0.1	0.0	1.2	-1.2	0.0	0.0	-5.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	6.0	3.4	0.1	0.0	0.0	1.2	0.0	0.0	0.0	28.7
Actual Evapotranspiration (AE)	2.2	1.2	2.1	2.7	4.9	6.6	6.0	3.8	5.6	3.2	0.3	1.0	39.6
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	1.5	3.3	0.0	0.9	2.0	0.1	7.8
Surplus (S)	2.6	1.9	2.4	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.4

1994	JAN	FEB	TOTAL
Precipitation (P)	4.2	2.3	6.5
Potential Evapotranspiration (PET)	1.5	2.1	3.6
P - PET	2.7	0.2	3.0
Change in Stored Soil Moisture (Δ ST)	2.7	0.2	2.9
Total Available Soil Moisture (ST)	2.7	2.9	5.6
Actual Evapotranspiration (AE)	1.5	2.1	3.6
Deficit (D)	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.0

30 Year Data	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.5	3.1	3.3	2.2	4.6	7.2	7.8	8.0	6.1	2.7	2.0	2.0	51.3
Potential Evapotranspiration (PET)	1.4	1.3	2.6	3.6	5.5	6.3	7.4	7.0	5.7	4.0	2.2	1.5	48.4
P - PET	1.1	1.8	0.7	-1.4	-1.0	0.9	0.4	1.0	0.4	-1.3	-0.2	0.5	2.9
Change in Stored Soil Moisture (Δ ST)	1.0	0.0	0.0	-1.4	-1.0	0.9	0.4	1.0	0.1	-1.3	-0.2	0.5	0.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	4.6	3.6	4.5	4.9	5.9	6.0	4.7	4.5	5.0	61.7
Actual Evapotranspiration (AE)	1.4	1.3	2.6	3.6	5.5	6.3	7.4	7.0	5.7	4.0	2.2	1.5	48.5
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.1	1.8	0.7	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	2.9

Table 5-10. Okeechobee HRCN Gate 6: Water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	1.4	3.3	1.8	2.5	1.1	18.8	4.1	7.5	4.3	2.5	3.3	0.7	51.2
Potential Evapotranspiration (PET)	3.0	4.2	5.1	5.9	6.5	7.3	8.3	7.3	6.5	4.9	3.8	3.0	65.8
P - PET	-1.7	-1.0	-3.2	-3.4	-5.4	11.5	-4.2	0.2	-2.2	-2.4	-0.5	-2.3	-14.6
Change in Stored Soil Moisture (Δ ST)	-1.7	-1.0	-0.3	0.0	0.0	6.0	-4.2	0.2	-2.0	0.0	0.0	0.0	-6.0
Total Available Soil Moisture (ST)	1.3	0.3	0.0	0.0	0.0	6.0	1.8	2.0	0.0	0.0	0.0	0.0	17.5
Actual Evapotranspiration (AE)	3.0	4.2	2.1	2.5	1.1	7.3	8.3	7.3	6.3	2.5	3.3	0.7	51.7
Deficit (D)	0.0	0.0	3.0	3.4	5.4	0.0	0.0	0.0	0.2	2.4	0.5	2.3	14.1
Surplus (S)	0.0	0.0	0.0	0.0	0.0	5.5	0.0	0.0	0.0	0.0	0.0	0.0	5.5

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	8.4	2.9	6.0	1.2	1.1	4.4	2.0	4.7	5.5	4.7	1.9	0.8	43.7
Potential Evapotranspiration (PET)	3.5	3.7	4.6	5.7	7.1	7.8	8.0	7.4	6.3	5.0	3.8	2.6	65.6
P - PET	4.9	-0.8	1.4	-4.5	-6.1	-3.4	-6.0	-2.7	-0.8	-0.3	-1.8	-1.8	-22.0
Change in Stored Soil Moisture (Δ ST)	4.9	-0.8	1.4	-4.5	-1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Available Soil Moisture (ST)	4.9	4.1	5.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.5
Actual Evapotranspiration (AE)	3.5	3.7	4.6	5.7	2.1	4.4	2.0	4.7	5.5	4.7	1.9	0.8	43.6
Deficit (D)	0.0	0.0	0.0	0.0	5.0	3.4	6.0	2.7	0.8	0.3	1.8	1.8	21.8
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1994	JAN	FEB	TOTAL
Precipitation (P)	3.2	4.4	7.6
Potential Evapotranspiration (PET)	3.1	4.3	7.3
P - PET	0.1	0.2	0.2
Change in Stored Soil Moisture (Δ ST)	0.1	0.2	0.3
Total Available Soil Moisture (ST)	0.1	0.3	0.4
Actual Evapotranspiration (AE)	3.1	4.3	7.4
Deficit (D)	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.0

Table 5-11. Okeechobee HRCN Gate 6: Water budgets calculated using a 6 inch model for soil moisture storage and using the Thornthwaite method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	1.4	3.3	1.8	2.5	1.1	18.8	4.1	7.5	4.3	2.5	3.3	0.7	51.2
Potential Evapotranspiration (PET)	1.5	2.1	3.1	3.6	4.4	6.5	10.1	7.6	6.7	4.3	3.1	1.9	55.0
P - PET	-0.2	1.1	-1.3	-1.1	-3.4	12.3	-6.0	-0.1	-2.5	-1.8	0.3	-1.3	-3.8
Change in Stored Soil Moisture (Δ ST)	-0.2	1.1	-1.3	-1.1	-1.5	6.0	-6.0	0.0	0.0	0.0	0.3	-0.3	-3.0
Total Available Soil Moisture (ST)	2.8	3.9	2.6	1.5	0.0	6.0	0.0	0.0	0.0	0.0	0.3	0.0	17.1
Actual Evapotranspiration (AE)	1.5	2.1	3.1	3.6	2.6	6.5	10.1	7.5	4.3	2.5	3.1	1.0	47.9
Deficit (D)	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.1	2.5	1.8	0.0	1.0	7.3
Surplus (S)	0.0	0.0	0.0	0.0	0.0	6.3	0.0	0.0	0.0	0.0	0.0	0.0	6.3

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	8.4	2.9	6.0	1.2	1.1	4.4	2.0	4.7	5.5	4.7	1.9	0.8	43.7
Potential Evapotranspiration (PET)	2.8	1.5	2.2	3.1	6.2	7.5	9.0	8.1	6.0	4.6	2.8	1.2	55.0
P - PET	5.7	1.5	3.8	-2.0	-5.1	-3.1	-7.0	-3.4	-0.5	0.2	-0.9	-0.4	-11.3
Change in Stored Soil Moisture (Δ ST)	5.7	0.3	0.0	-2.0	-4.0	0.0	0.0	0.0	0.0	0.2	-0.2	0.0	0.0
Total Available Soil Moisture (ST)	5.7	6.0	6.0	4.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	21.9
Actual Evapotranspiration (AE)	2.8	1.5	2.2	3.1	5.1	4.4	2.0	4.7	5.5	4.6	2.1	0.8	38.8
Deficit (D)	0.0	0.0	0.0	0.0	1.1	3.1	7.0	3.4	0.5	0.0	0.7	0.4	16.2
Surplus (S)	0.0	1.2	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0

1994	JAN	FEB	TOTAL
Precipitation (P)	3.2	4.4	7.6
Potential Evapotranspiration (PET)	1.6	2.5	4.1
P - PET	1.6	1.9	3.5
Change in Stored Soil Moisture (Δ ST)	1.6	1.9	3.5
Total Available Soil Moisture (ST)	1.6	3.5	5.1
Actual Evapotranspiration (AE)	1.6	2.5	4.1
Deficit (D)	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.0

Table 5-12. Archbold Biological Station: Water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	0.4	4.7	2.3	4.9	3.8	15.8	4.7	12.1	6.7	1.9	4.4	0.6	82.2
Potential Evapotranspiration (PET)	2.7	3.8	4.5	5.4	6.1	7.4	7.5	7.0	6.2	4.5	3.8	2.8	61.8
P - PET	-2.4	0.9	-2.3	-0.5	-2.2	8.3	-2.8	5.1	0.6	-2.6	0.6	-2.2	0.4
Change in Stored Soil Moisture (Δ ST)	-2.4	0.9	-1.5	0.0	0.0	6.0	-2.8	2.8	0.0	-2.6	0.6	-2.2	-1.2
Total Available Soil Moisture (ST)	0.6	1.5	0.0	0.0	0.0	6.0	3.2	6.0	6.0	3.4	4.0	1.8	32.5
Actual Evapotranspiration (AE)	2.7	3.8	3.8	4.9	3.8	7.4	7.5	7.0	6.2	4.5	3.8	2.8	58.2
Deficit (D)	0.0	0.0	0.7	0.5	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4
Surplus (S)	0.0	0.0	0.0	0.0	0.0	2.3	0.0	2.3	0.6	0.0	0.0	0.0	5.2

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.1	3.1	5.7	2.8	1.1	5.0	11.0	4.3	4.6	7.0	0.8	1.3	51.8
Potential Evapotranspiration (PET)	3.3	3.3	4.3	5.2	6.5	7.4	7.5	7.0	6.2	4.9	3.5	2.4	61.6
P - PET	1.8	-0.3	1.4	-2.4	-5.4	-2.5	3.6	-2.7	-1.5	2.0	-2.7	-1.1	-9.8
Change in Stored Soil Moisture (Δ ST)	1.8	-0.3	1.4	-2.4	-2.3	0.0	3.6	-2.7	-0.9	2.0	-2.0	0.0	-1.8
Total Available Soil Moisture (ST)	3.6	3.3	4.7	2.3	0.0	0.0	3.6	0.9	0.0	2.0	0.0	0.0	20.4
Actual Evapotranspiration (AE)	3.3	3.3	4.3	5.2	3.4	5.0	7.5	7.0	5.5	4.9	2.8	1.3	53.5
Deficit (D)	0.0	0.0	0.0	0.0	3.1	2.5	0.0	0.0	0.6	0.0	0.7	1.1	8.0
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

1993	JAN	FEB	TOTAL
Precipitation (P)	3.8	1.8	5.7
Potential Evapotranspiration (PET)	2.9	3.9	6.8
P - PET	1.0	-2.1	-1.1
Change in Stored Soil Moisture (Δ ST)	1.0	-1.0	0.0
Total Available Soil Moisture (ST)	1.0	0.0	1.0
Actual Evapotranspiration (AE)	2.9	2.8	5.7
Deficit (D)	0.0	1.1	1.1
Surplus (S)	0.0	0.0	0.0

30 Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.1	2.5	2.8	2.3	4.3	8.7	7.6	7.5	6.8	4.0	1.7	1.8	51.9
Potential Evapotranspiration (PET)	2.8	3.6	4.7	5.8	6.9	7.4	7.5	7.1	6.2	4.8	3.5	2.7	63.1
P - PET	-0.8	-1.1	-2.0	-3.5	-2.6	1.3	0.1	0.4	0.6	-0.9	-1.8	-0.9	-11.1
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	0.0	0.0	1.3	0.1	0.4	0.6	-0.9	-1.5	0.0	0.0
Total Available Soil Moisture (ST)	0.0	0.0	0.0	0.0	0.0	1.3	1.4	1.8	2.4	1.5	0.0	0.0	8.4
Actual Evapotranspiration (AE)	2.1	2.5	2.8	2.3	4.3	7.4	7.5	7.1	6.2	4.8	3.2	1.8	51.9
Deficit (D)	0.8	1.1	2.0	3.5	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 5-13. Archbold Biological Station: Water budgets calculated using a 6 inch model for soil moisture storage and using the Thornthwaite method for estimating PET. All values are in inches.

1992	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	0.4	4.7	2.3	4.9	3.8	15.8	4.7	12.1	6.7	1.9	4.4	0.6	62.2
Potential Evapotranspiration (PET)	0.6	2.0	2.6	3.2	4.1	6.2	6.8	6.6	5.6	3.7	3.2	2.0	46.6
P - PET	-0.2	2.7	-0.4	1.7	-0.3	9.6	-2.2	5.5	1.2	-1.7	1.1	-1.4	15.6
Change in Stored Soil Moisture (Δ ST)	-0.2	0.2	-0.4	0.4	-0.3	0.3	-2.2	2.2	0.0	-1.7	1.1	-1.4	-2.0
Total Available Soil Moisture (ST)	5.8	6.0	5.6	6.0	5.7	6.0	3.8	6.0	6.0	4.3	5.4	4.0	64.6
Actual Evapotranspiration (AE)	0.6	2.0	2.6	3.2	4.1	6.2	6.8	6.6	5.6	3.7	3.2	2.0	46.6
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.0	2.5	0.0	1.3	0.0	9.3	0.0	3.3	1.2	0.0	0.0	0.0	17.6

1993	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	5.1	3.1	5.7	2.8	1.1	5.0	11.0	4.3	4.6	7.0	0.8	1.3	51.8
Potential Evapotranspiration (PET)	2.7	1.4	2.3	2.8	4.8	6.2	6.9	6.6	5.6	4.5	2.7	1.4	48.0
P - PET	2.5	1.6	3.4	-0.1	-3.8	-1.2	4.1	-2.3	-0.9	2.4	-1.9	-0.1	3.8
Change in Stored Soil Moisture (Δ ST)	2.0	0.0	0.0	-0.1	-3.8	-1.2	4.1	-2.3	-0.9	2.4	-1.9	-0.1	-1.8
Total Available Soil Moisture (ST)	6.0	6.0	6.0	5.9	2.1	0.9	5.0	2.7	1.8	4.2	2.3	2.2	45.1
Actual Evapotranspiration (AE)	2.7	1.4	2.3	2.8	4.8	6.2	6.9	6.6	5.6	4.5	2.7	1.4	47.9
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.5	1.6	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5

1994	JAN	FEB	TOTAL
Precipitation (P)	3.8	1.8	5.7
Potential Evapotranspiration (PET)	1.6	1.9	3.5
P - PET	2.2	-0.0	2.2
Change in Stored Soil Moisture (Δ ST)	2.2	0.0	2.2
Total Available Soil Moisture (ST)	4.4	4.4	8.8
Actual Evapotranspiration (AE)	1.6	1.9	3.5
Deficit (D)	0.0	0.0	0.0
Surplus (S)	0.0	0.0	0.0

30 Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.1	2.5	2.8	2.3	4.3	8.7	7.6	7.5	6.8	4.0	1.7	1.8	51.9
Potential Evapotranspiration (PET)	1.4	1.4	2.6	3.6	5.5	6.2	7.1	6.9	5.7	4.2	2.3	1.5	48.6
P - PET	0.6	1.1	0.1	-1.3	-1.2	2.5	0.4	0.6	1.1	-0.2	-0.7	0.3	3.3
Change in Stored Soil Moisture (Δ ST)	0.6	0.0	0.0	-1.3	-1.2	2.5	0.0	0.0	0.0	-0.2	-0.7	0.3	0.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	4.7	3.5	6.0	6.0	6.0	6.0	5.8	5.1	5.4	66.5
Actual Evapotranspiration (AE)	1.4	1.4	2.6	3.6	5.5	6.2	7.1	6.9	5.7	4.2	2.3	1.5	48.4
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.0	1.1	0.1	0.0	0.0	0.0	0.4	0.6	1.1	0.0	0.0	0.0	3.3

Table 5-14. Stuart 1 N: Thirty-year water budgets calculated using a 6 inch model for soil moisture storage and using the Stephens-Stewart and the Thornthwaite method for estimating PET. All values are in inches.

Stephens-Stewart Method

30 Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.6	2.6	3.2	2.7	5.0	7.0	6.5	5.8	7.6	6.8	2.5	2.6	54.9
Potential Evapotranspiration (PET)	3.1	3.8	4.9	5.9	6.9	7.5	7.5	7.1	6.3	5.0	3.7	2.9	64.6
P - PET	-0.5	-1.2	-1.7	-3.3	-1.9	-0.5	-1.1	-1.4	1.4	1.8	-1.1	-0.3	-9.7
Change in Stored Soil Moisture (Δ ST)	-0.5	-1.2	-0.1	0.0	0.0	0.0	0.0	0.0	1.4	1.8	-1.1	-0.3	0.0
Total Available Soil Moisture (ST)	1.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	1.4	3.2	2.1	1.8	9.9
Actual Evapotranspiration (AE)	3.1	3.8	3.3	2.7	5.0	7.0	6.5	5.8	6.3	5.0	3.7	2.9	55.1
Deficit (D)	0.0	0.0	1.6	3.3	1.9	0.5	1.1	1.4	0.0	0.0	0.0	0.0	9.8
Surplus (S)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Thornthwaite Method

30 Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	TOTAL
Precipitation (P)	2.6	2.6	3.2	2.7	5.0	7.0	6.5	5.8	7.6	6.8	2.5	2.6	54.9
Potential Evapotranspiration (PET)	1.7	1.6	2.8	3.7	5.5	6.3	7.3	7.2	6.0	4.5	2.7	1.9	51.2
P - PET	0.9	1.0	0.3	-1.0	-0.6	0.7	-0.9	-1.4	1.7	2.3	-0.1	0.8	3.6
Change in Stored Soil Moisture (Δ ST)	0.0	0.0	0.0	-1.0	-0.6	0.7	-0.9	-1.4	1.7	1.5	-0.1	0.1	0.0
Total Available Soil Moisture (ST)	6.0	6.0	6.0	5.0	4.4	5.1	4.2	2.8	4.5	6.0	5.9	6.0	61.9
Actual Evapotranspiration (AE)	1.7	1.6	2.8	3.7	5.5	6.3	7.3	7.2	6.0	4.5	2.7	1.9	51.2
Deficit (D)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Surplus (S)	0.9	1.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.7	3.7

Figure 5-1. Numbers of snails found by timed and quadrant collection methods and estimated soil surface moisture for snail habitats at the University of Florida, Dairy Research Unit site 1 (a,b) and site 2 (c,d). S, saturated/submerged; W, wet; M, moist; D, dry. Missing soil moisture data indicated by *.

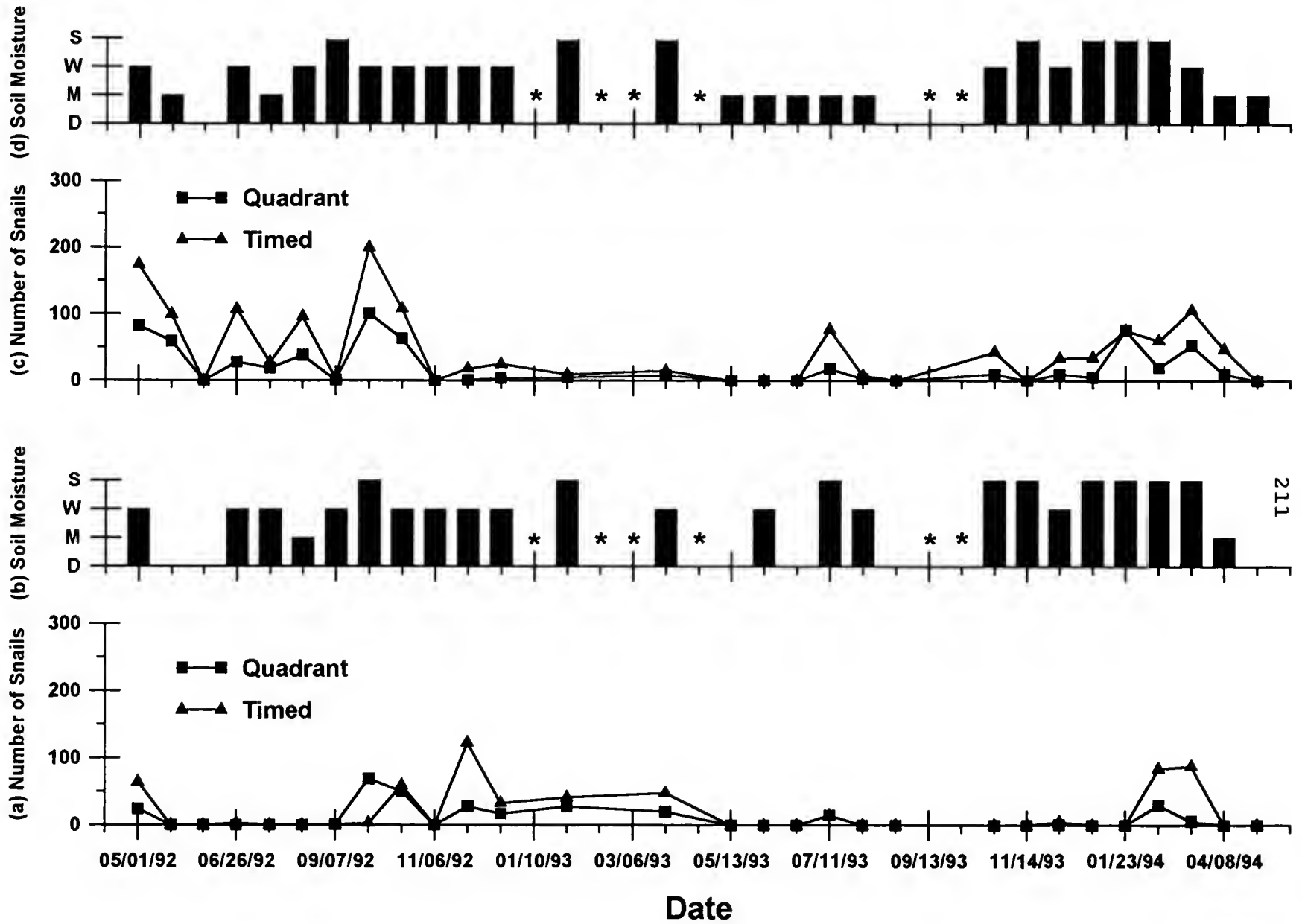

(d) Soil Moisture
S
W
M
D
(c) Number of Snails
300
200
100
0
Quadrant
Timed
(b) Soil Moisture
S
W
M
D
(a) Number of Snails
300
200
100
0
Quadrant
Timed
05/01/92
06/26/92
09/07/92
11/06/92
01/10/93
03/06/93
05/13/93
07/11/93
09/13/93
11/14/93
01/23/94
04/08/94
Date

Figure 5-2. Numbers of snails found by timed and quadrant collection methods and estimated soil surface moisture for snail habitats at Deseret Ranch, site 2 (a,b) and H. E. Wolfe Ranch, site 2 (c,d). S, saturated/submerged; W, wet; M, moist; D, dry. Missing soil moisture data indicated by *.

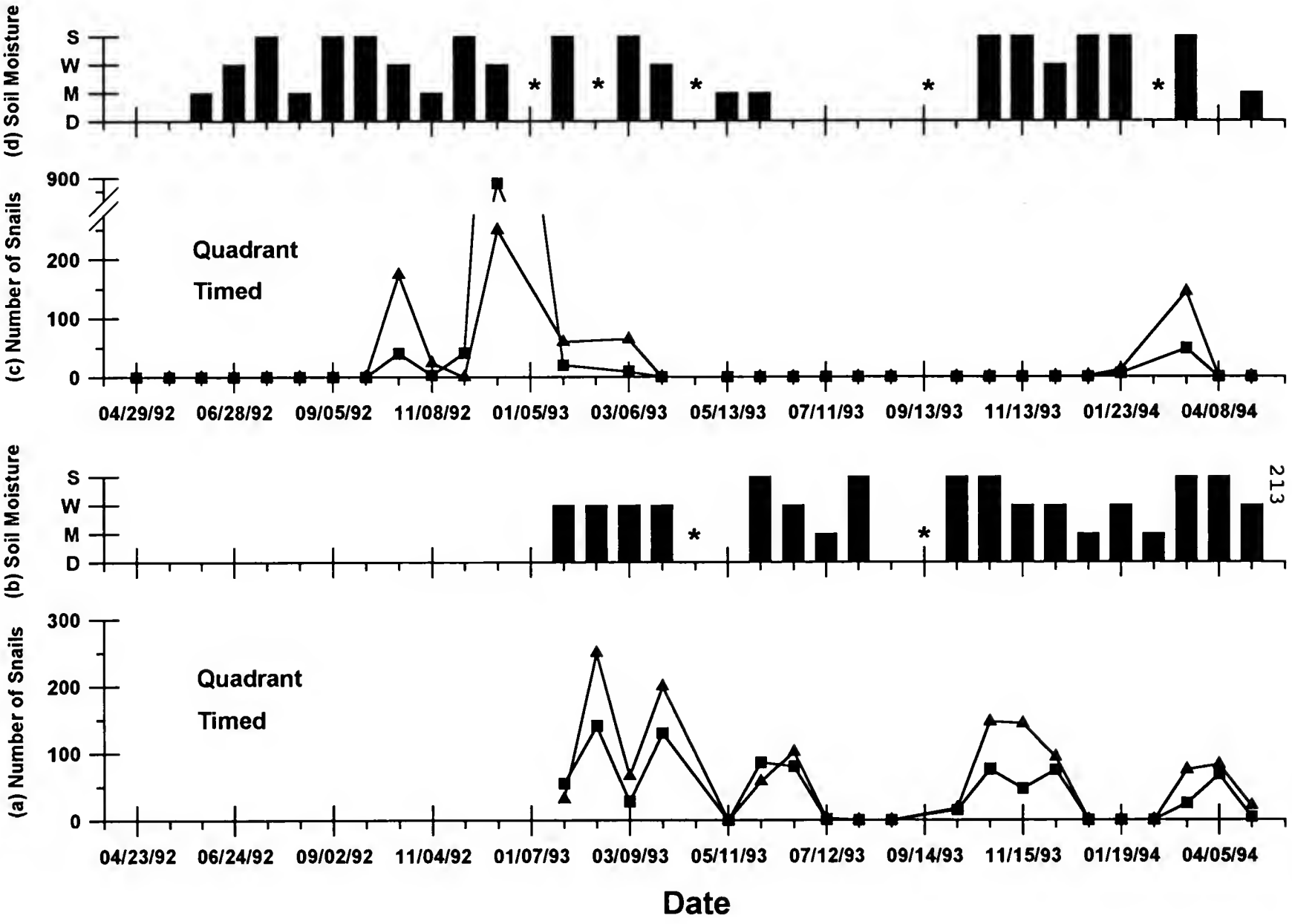

(d) Soil Moisture
S
W
M
D
(c) Number of Snails
900
200
100
0
Quadrant
Timed
04/29/92
06/28/92
09/05/92
11/08/92
01/05/93
03/06/93
05/13/93
07/11/93
09/13/93
11/13/93
01/23/94
04/08/94
(b) Soil Moisture
S
W
M
D
(a) Number of Snails
300
200
100
0
Quadrant
Timed
04/23/92
06/24/92
09/02/92
11/04/92
01/07/93
03/09/93
05/11/93
07/12/93
09/14/93
11/15/93
01/19/94
04/05/94
Date

Figure 5-3. Numbers of snails found by timed and quadrant collection methods and estimated soil surface moisture for snail habitats at Creek Ranch. Site 1 (a,b) and site 2 (c,d). S, saturated/submerged; W, wet; M, moist; D, dry. Missing soil moisture data indicated by *.

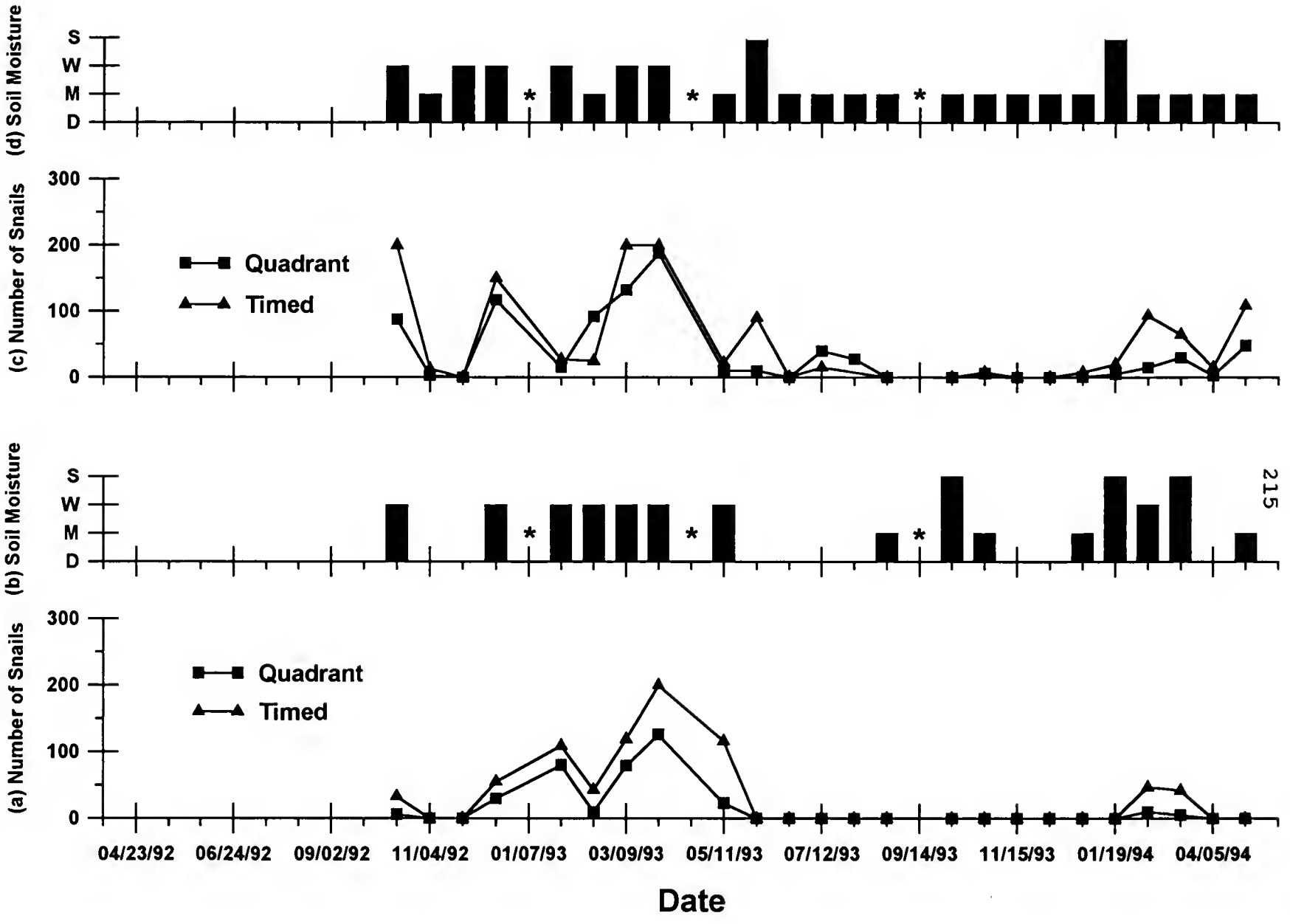
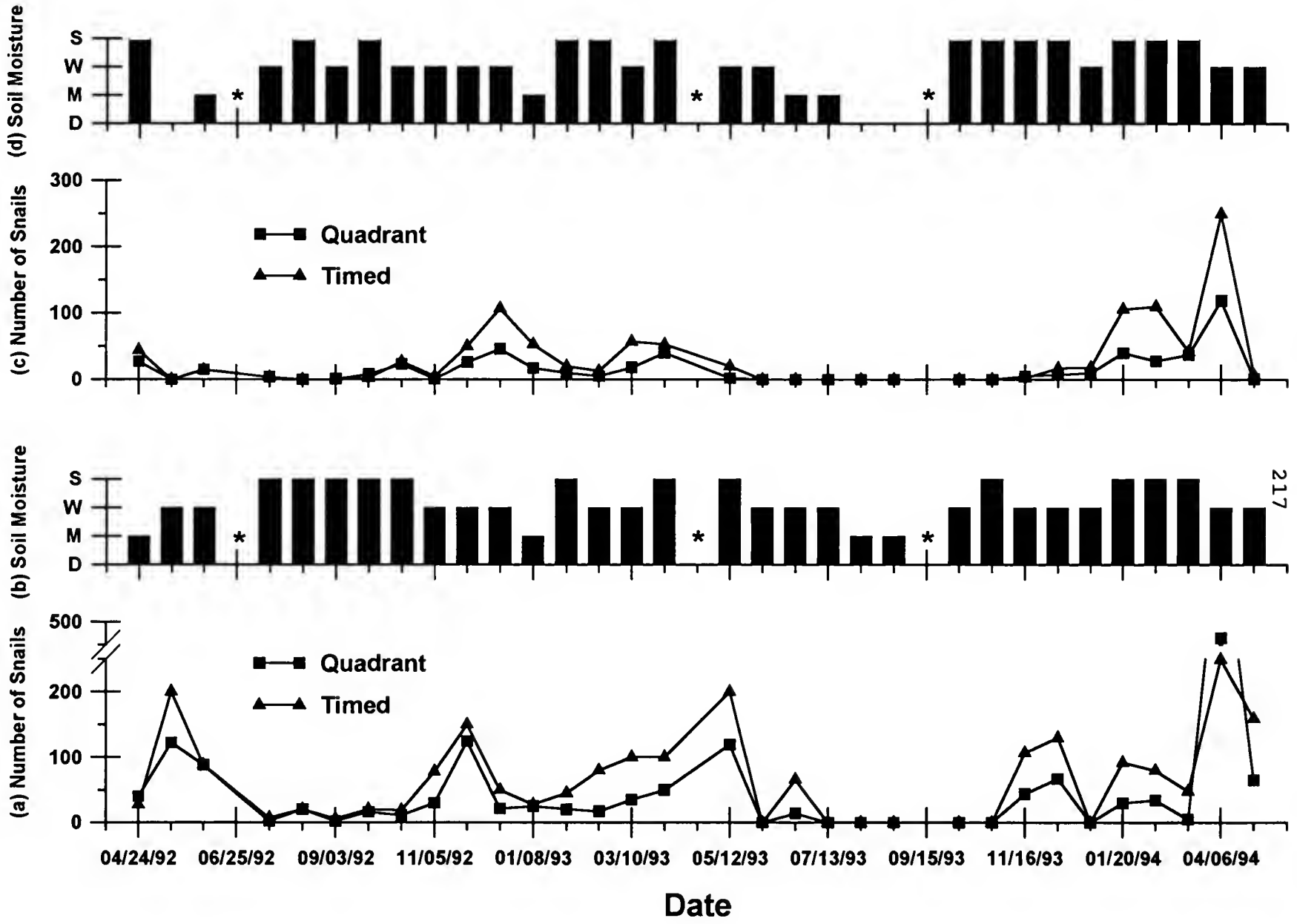

(d) Soil Moisture
S
W
M
D
(c) Number of Snails
300
200
100
0
Quadrant
Timed
(b) Soil Moisture
S
W
M
D
(a) Number of Snails
300
200
100
0
Quadrant
Timed
04/23/92
06/24/92
09/02/92
11/04/92
01/07/93
03/09/93
05/11/93
07/12/93
09/14/93
11/15/93
01/19/94
04/05/94
Date

Figure 5-4. Numbers of snails found by timed and quadrant collection methods and estimated soil surface moisture for snail habitats at the Brighton Seminole Indian Reservation site 1 (a,b) and site 2 (c,d). S, saturated/submerged; W, wet; M, moist; D, dry. Missing soil moisture data indicated by *.


(d) Soil Moisture
S
W
M
D
(c) Number of Snails
300
200
100
0
Quadrant
Timed
(b) Soil Moisture
S
W
M
D
(a) Number of Snails
500
200
100
0
Quadrant
Timed
04/24/92
06/25/92
09/03/92
11/05/92
01/08/93
03/10/93
05/12/93
07/13/93
09/15/93
11/16/93
01/20/94
04/06/94
Date

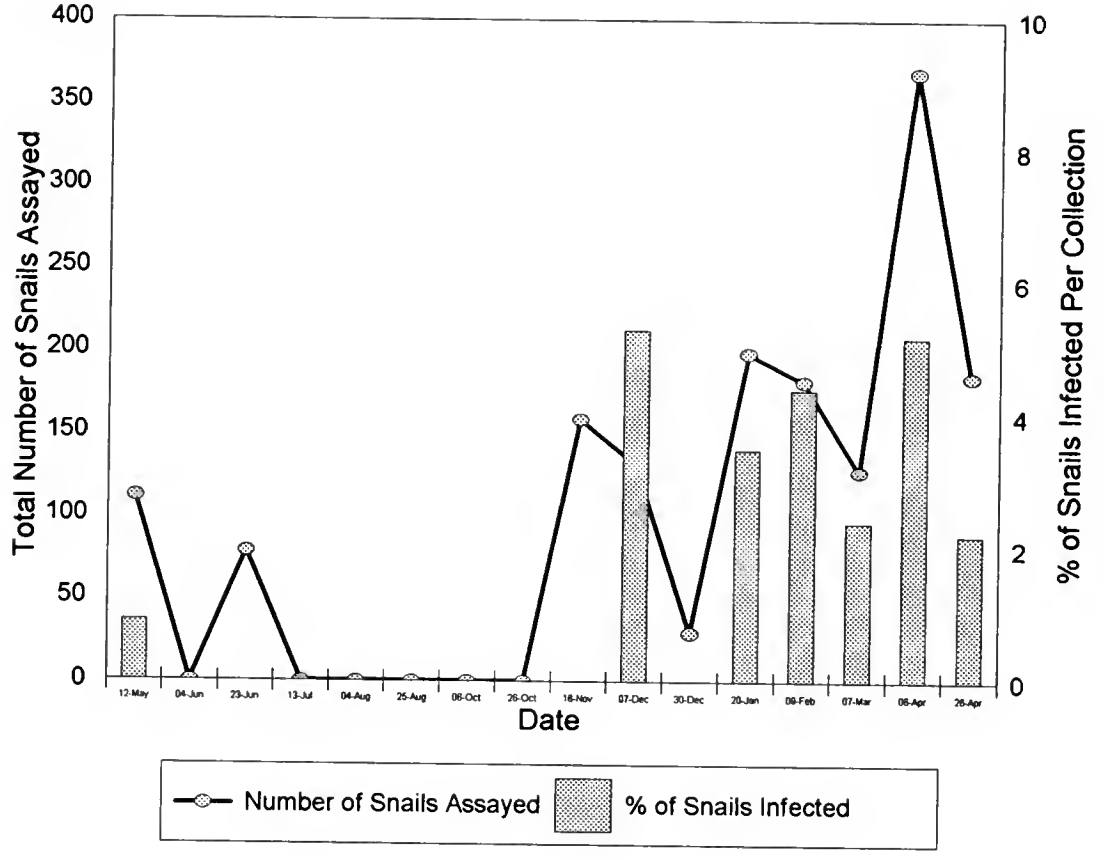
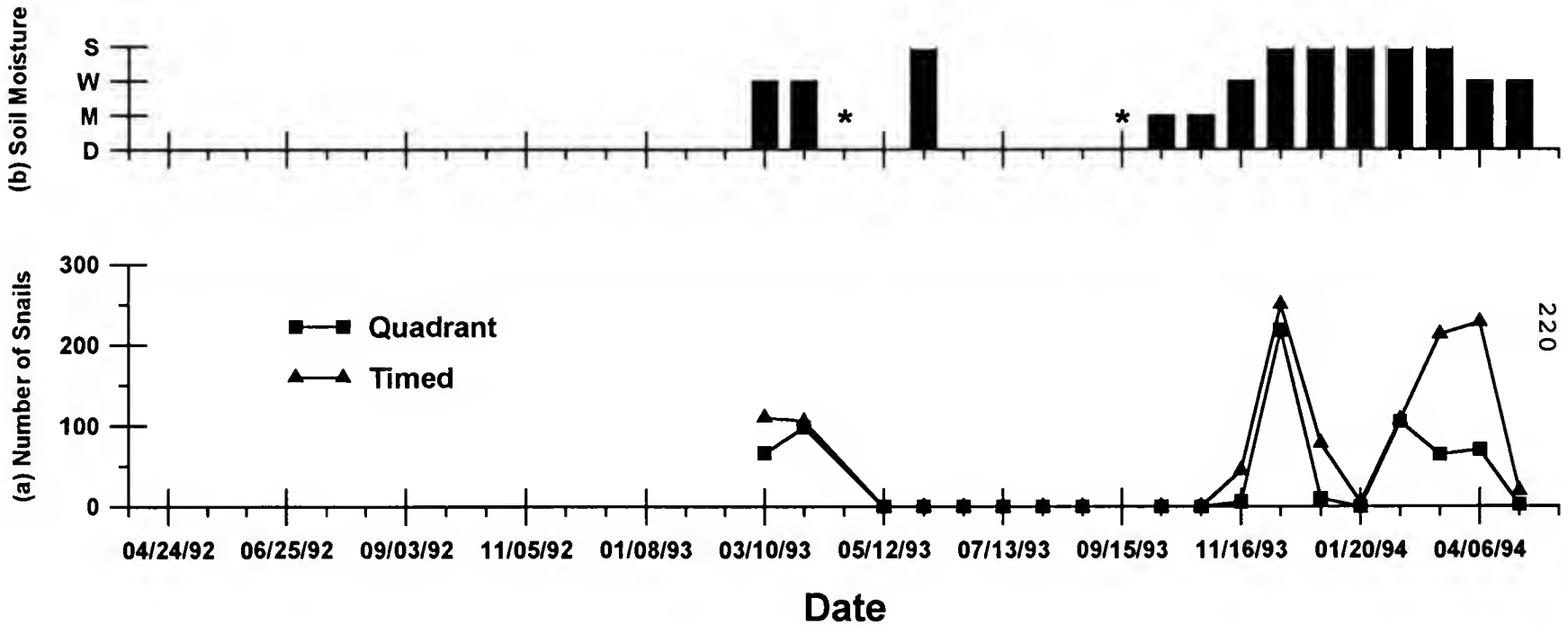


Figure 5-5 Brighton Seminole Indian Reservation: Numbers of snails assayed for infection with the DNA probe, and the percent of snails infected per collection during the second year of the study.

Figure 5-6. Numbers of snails found by timed and quadrant collection methods and estimated soil surface moisture for snail habitats at Williams Ranch, site 1. S, saturated/submerged; W, wet; M, moist; D, dry. Missing soil moisture data indicated by *.


(b) Soil Moisture
S
W
M
D
*
*
(a) Number of Snails
300
200
100
0
Quadrant
Timed
220
04/24/92
06/25/92
09/03/92
11/05/92
01/08/93
03/10/93
05/12/93
07/13/93
09/15/93
11/16/93
01/20/94
04/06/94
Date

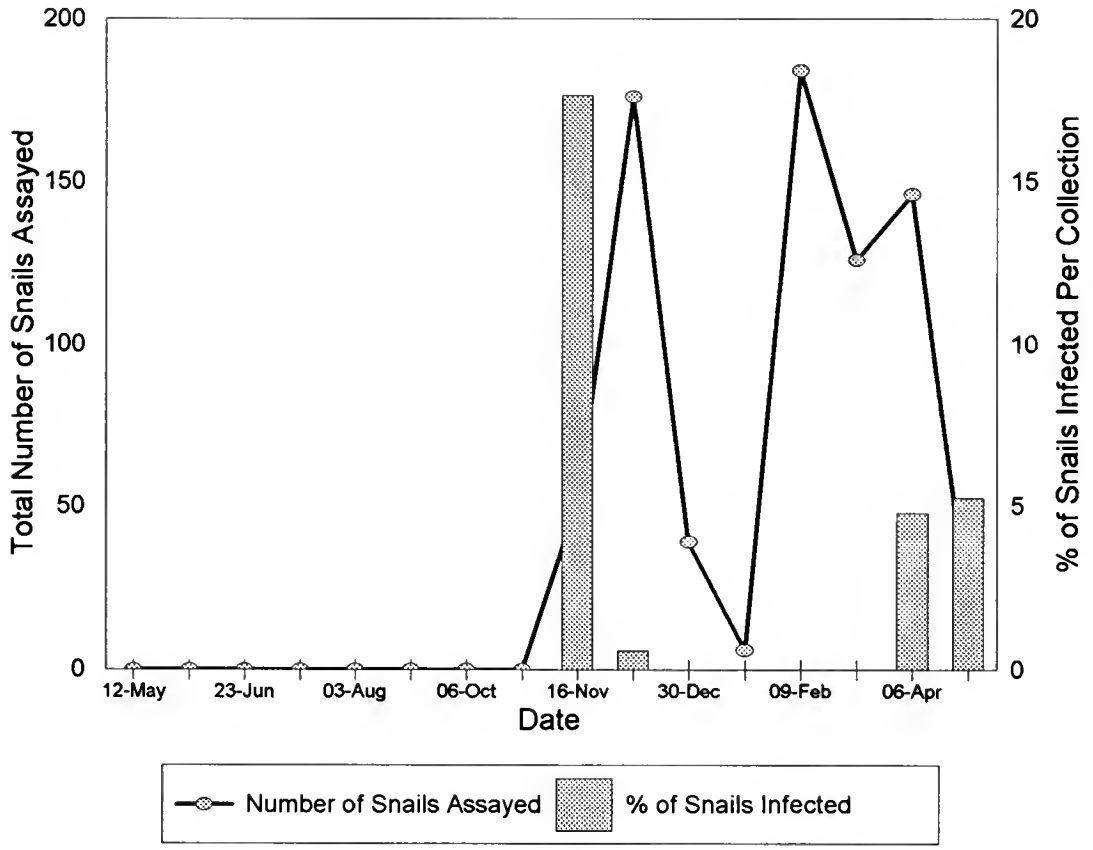
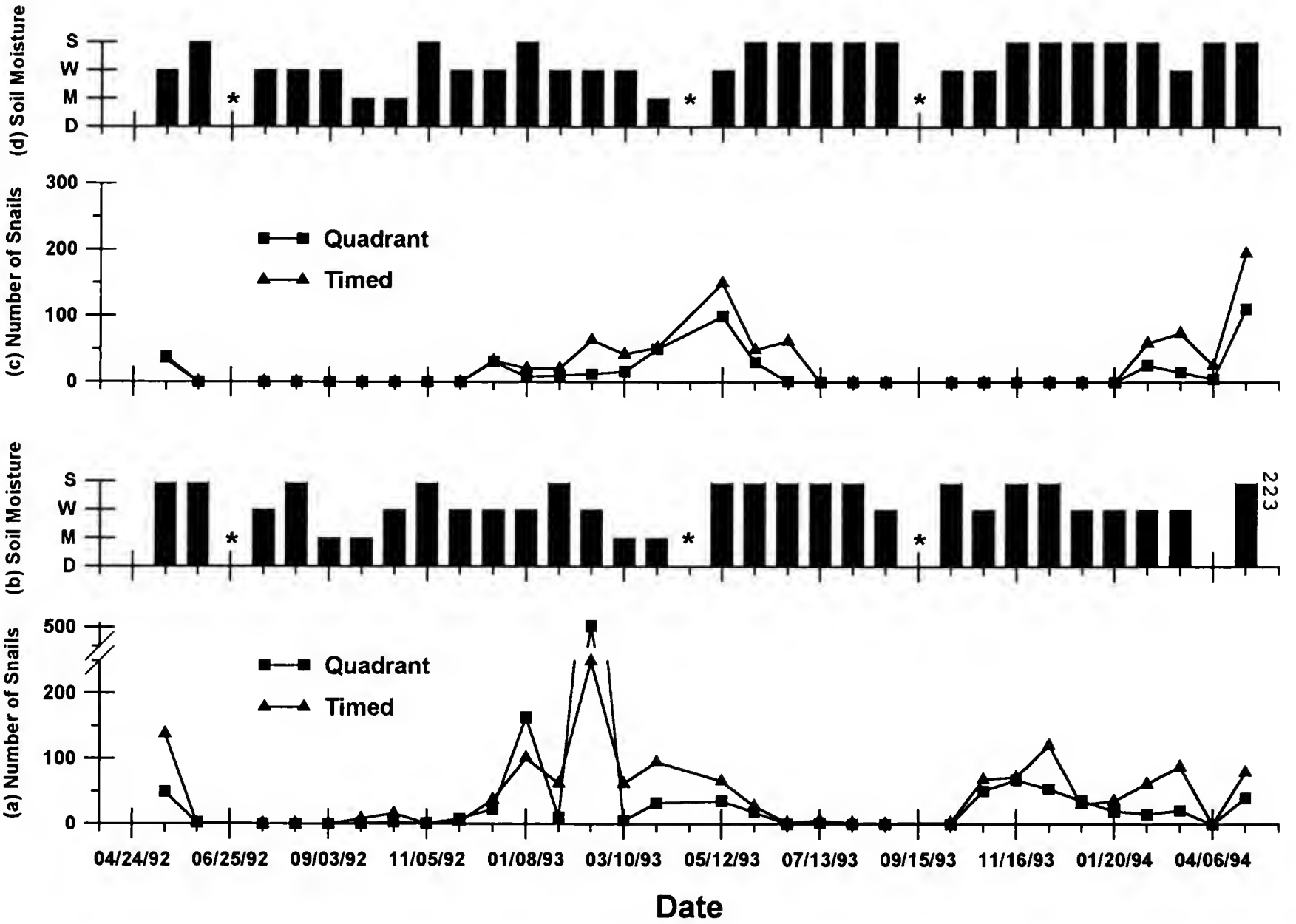


Figure 5-7 Williams Ranch: Numbers of snails assayed for infection with the DNA probe, and the percent of snails infected per collection during the second year of the study.

Figure 5-8. Numbers of snails found by timed and quadrant collection methods and estimated soil surface moisture for snail habitats at Rio Ranch site 1 (a,b) and site 2 (c,d). S, saturated/submerged; W, wet; M, moist; D, dry. Missing soil moisture data indicated by *.


(d) Soil Moisture
S
W
M
D
*
*
*
(c) Number of Snails
300
200
100
0
Quadrant
Timed
(b) Soil Moisture
S
W
M
D
*
*
*
(a) Number of Snails
500
200
100
0
Quadrant
Timed
04/24/92
06/25/92
09/03/92
11/05/92
01/08/93
03/10/93
05/12/93
07/13/93
09/15/93
11/16/93
01/20/94
04/06/94
Date

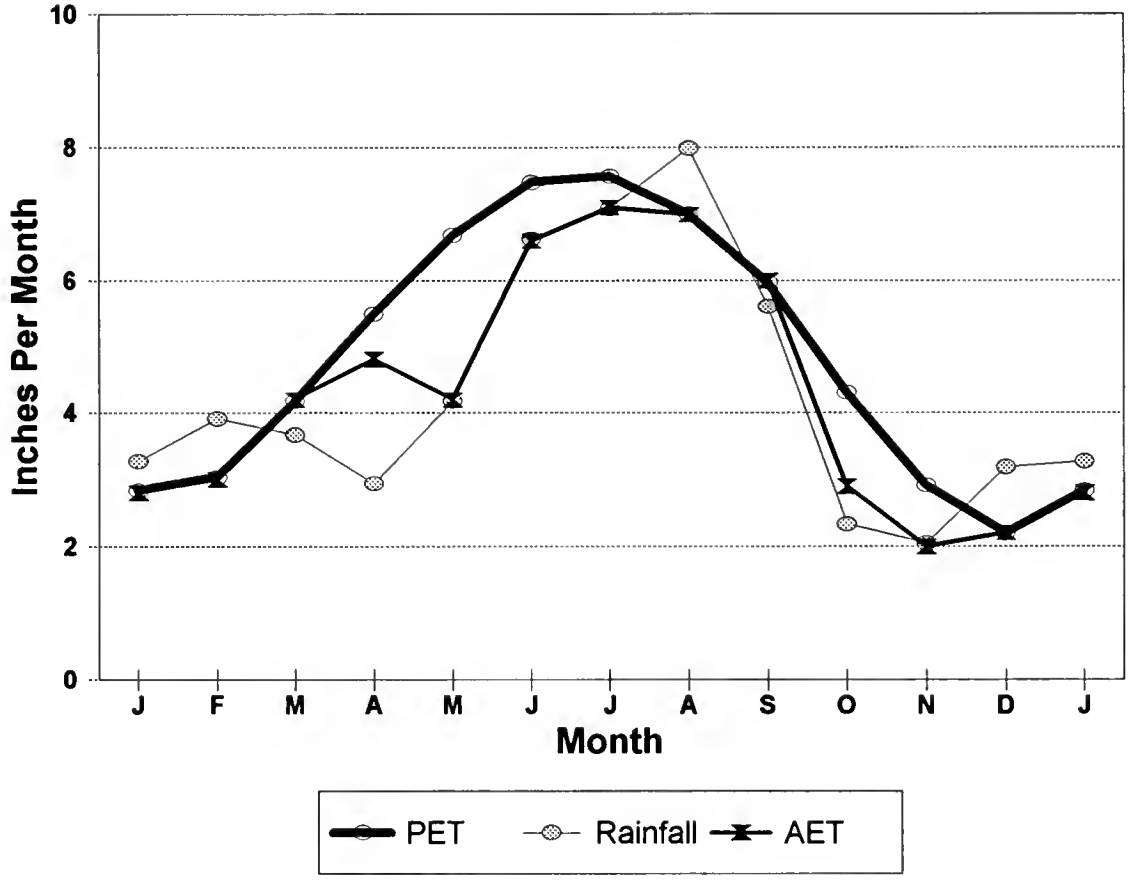


Figure 5-9. Thirty year water balance for Gainesville 2 WSW (02-3321) using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

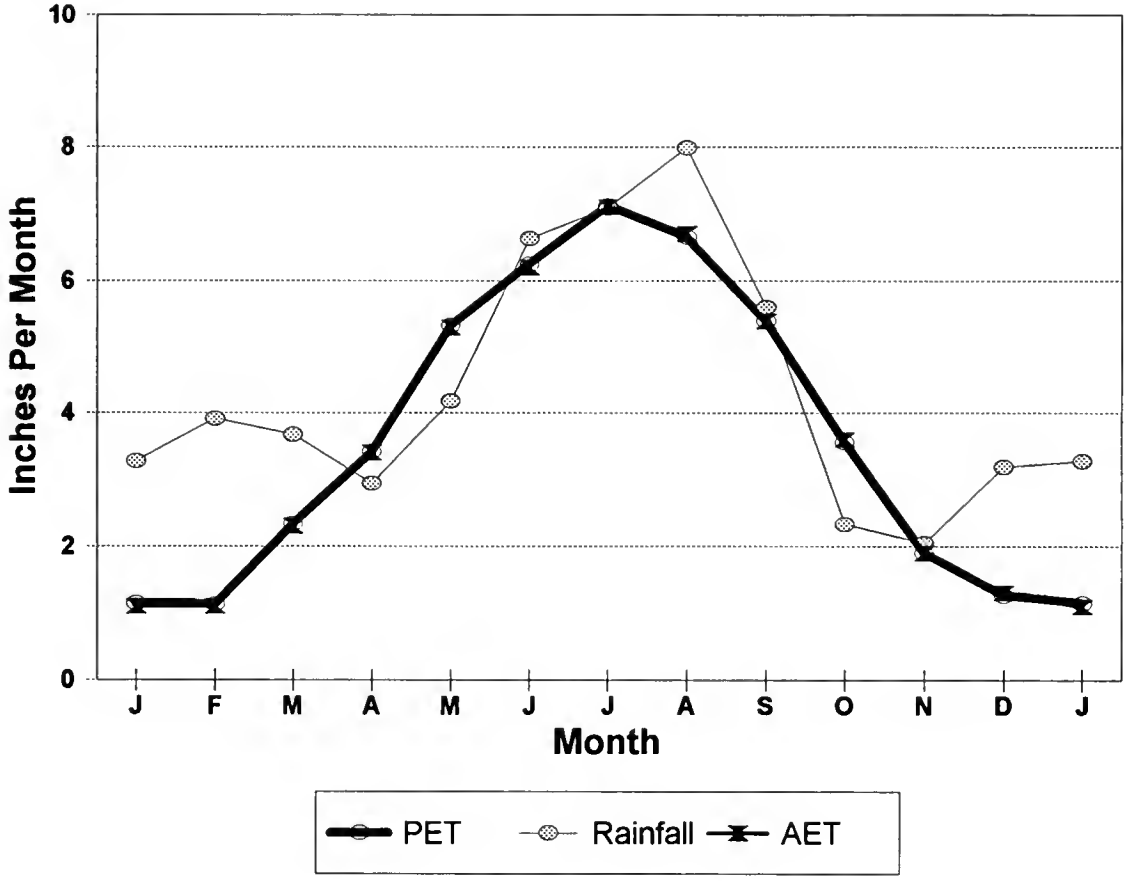


Figure 5-10. Thirty year water balance for Gainesville 2 WSW (02-3321) using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

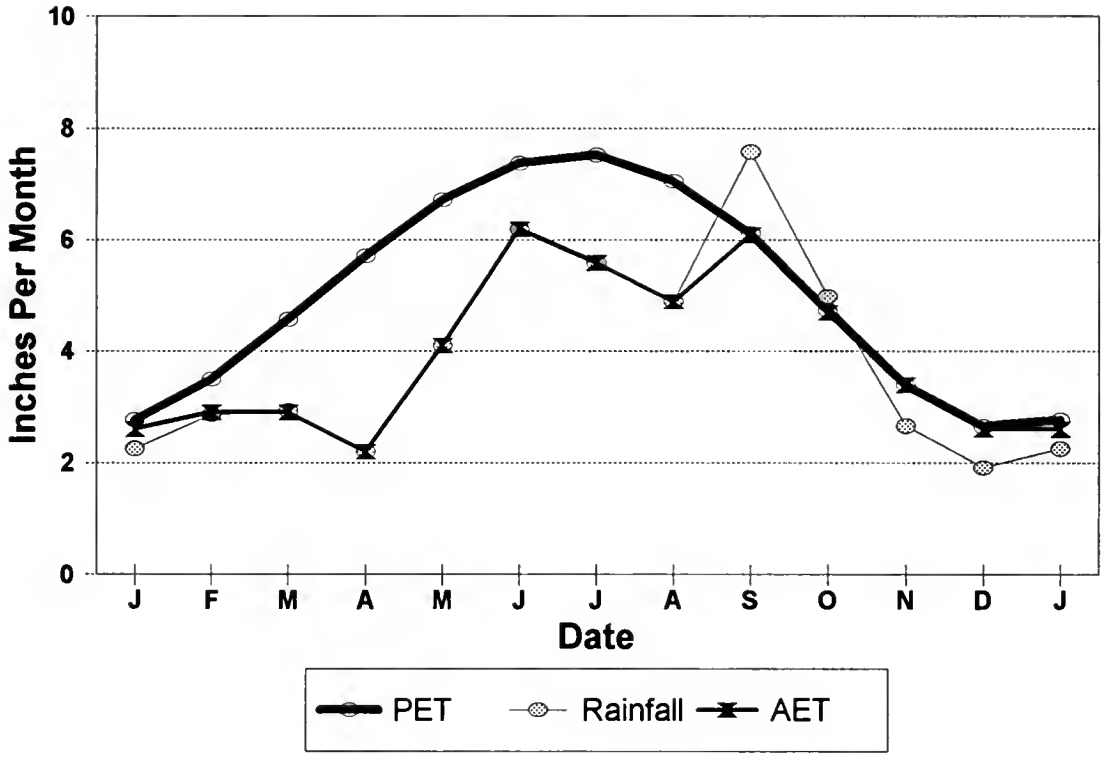


Figure 5-11. Thirty year water balance for Melbourne WSO (04-5612) using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

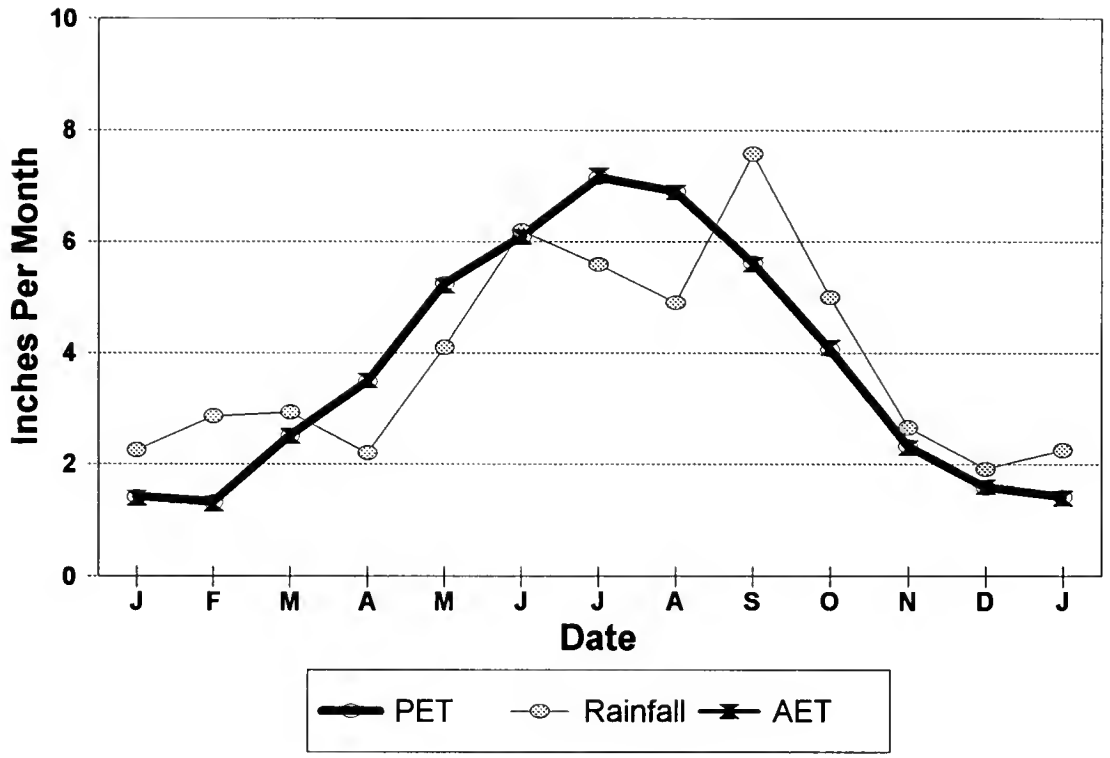


Figure 5-12. Thirty year water balance for Melbourne WSO (04-5612) using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

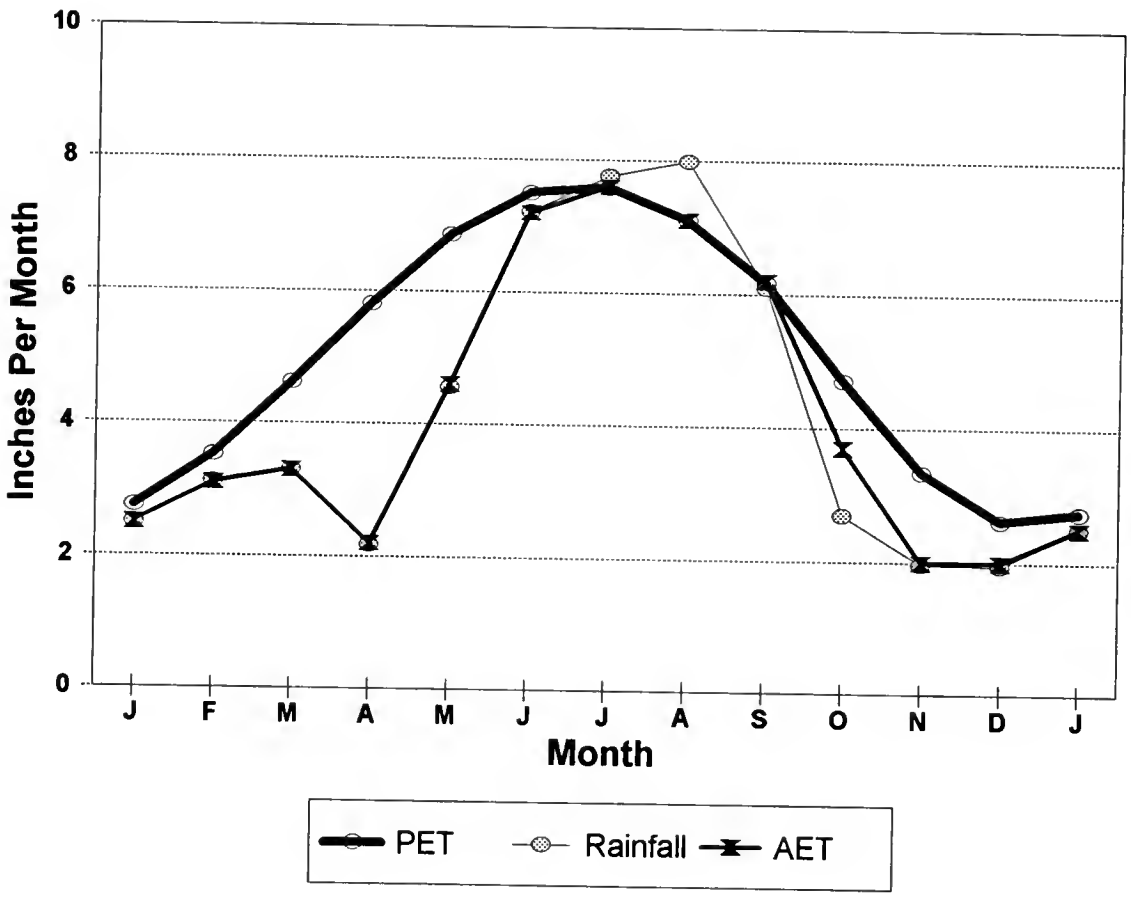


Figure 5-13. Thirty year water balance for Mountain Lake (04-5973) using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

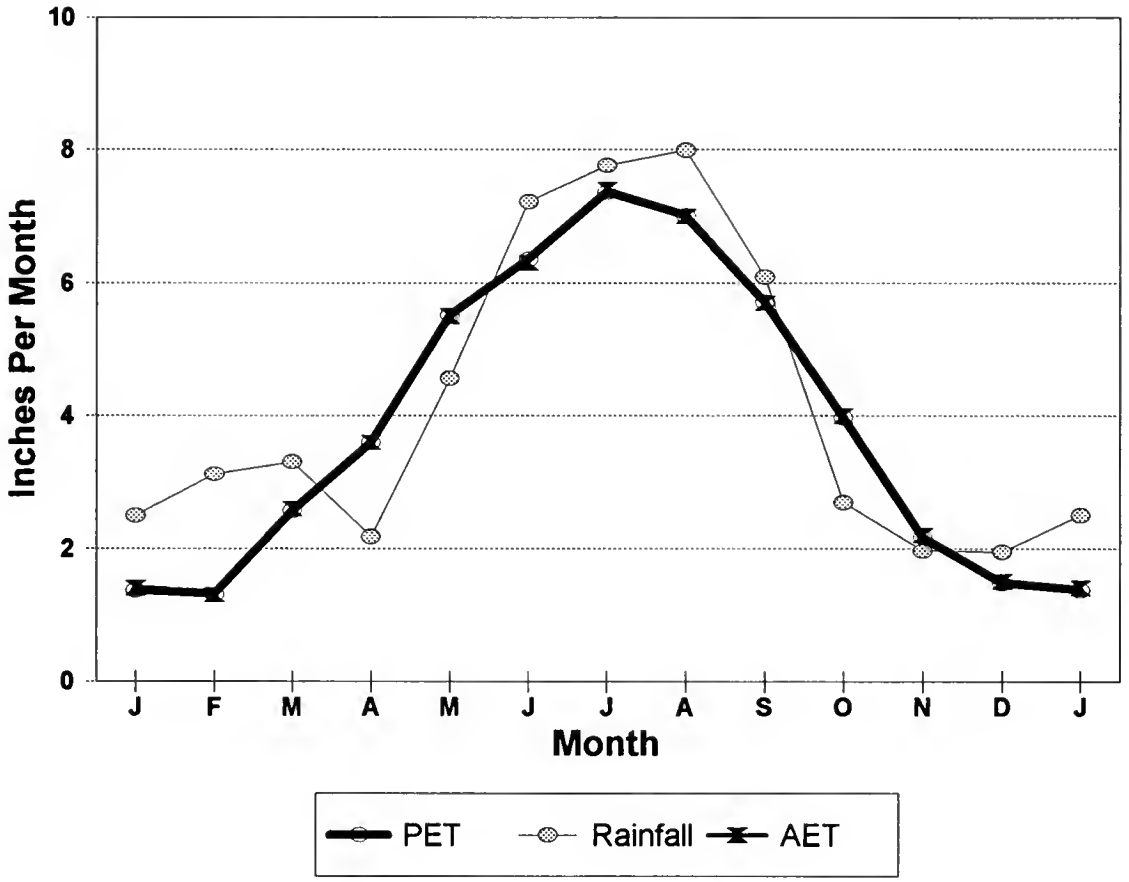


Figure 5-14. Thirty year water balance for Mountain Lake (04-5973) using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

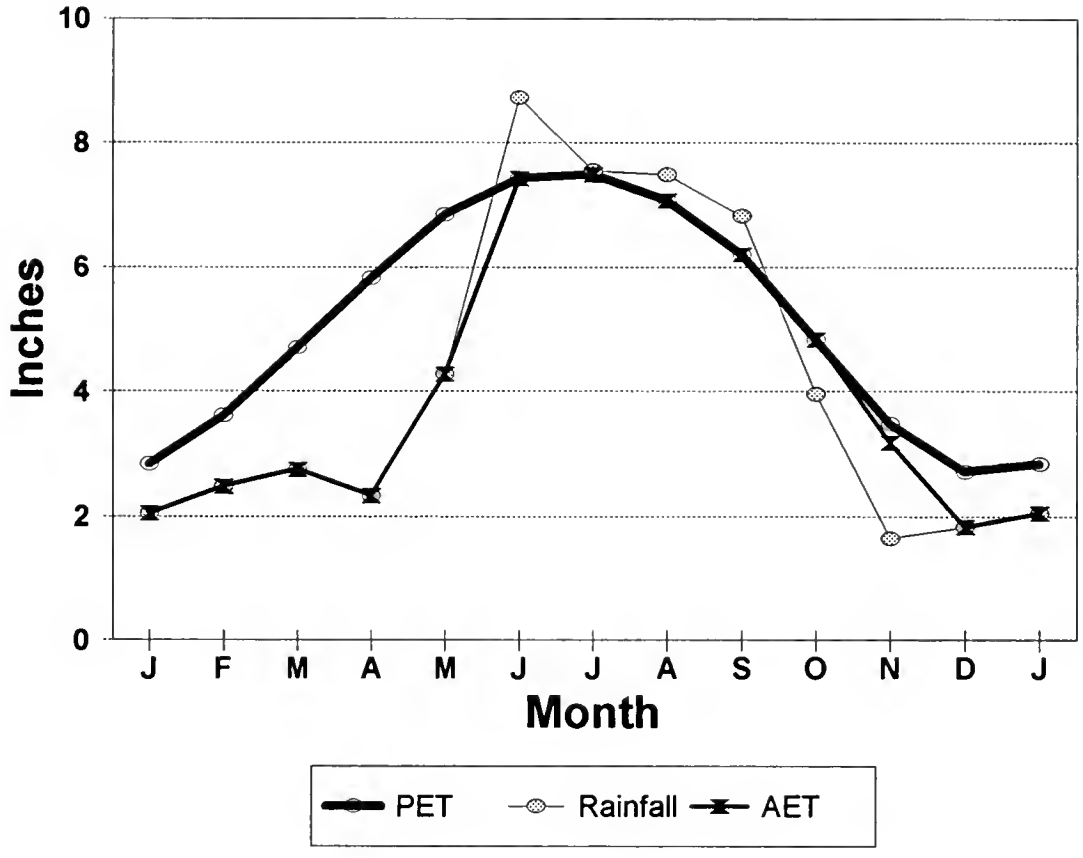


Figure 5-15. Thirty year water balance for Archbold Biological Station (04-0236) using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

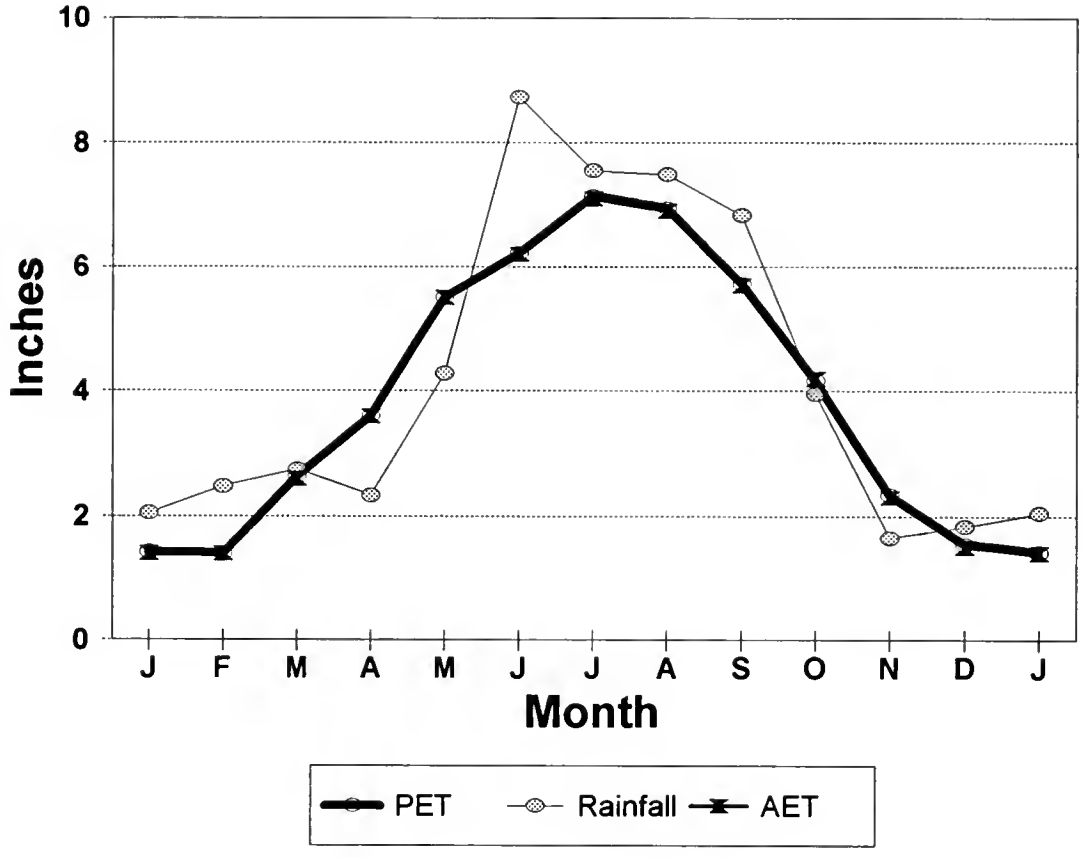


Figure 5-16. Thirty year water balance for Archbold Biological Station (04-0236) using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

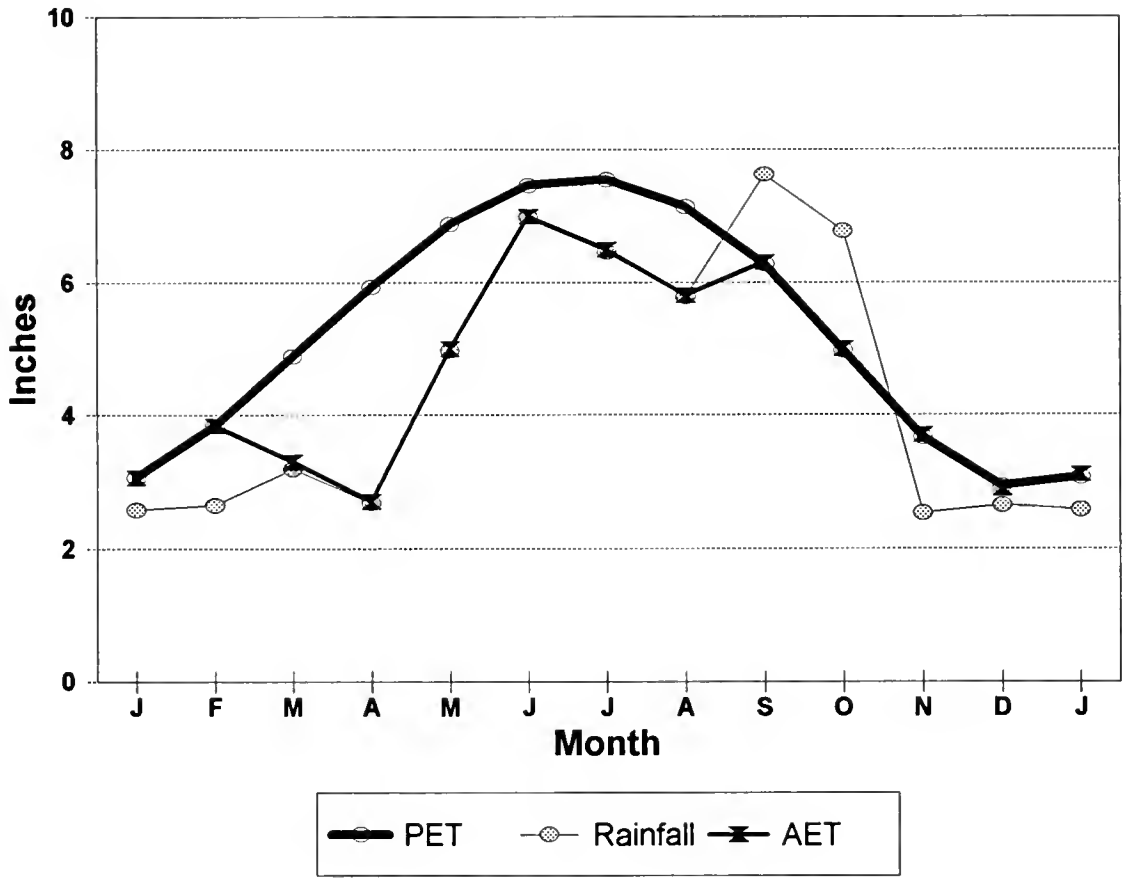


Figure 5-17. Thirty year water balance for Stuart 1 N (06-8620) using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

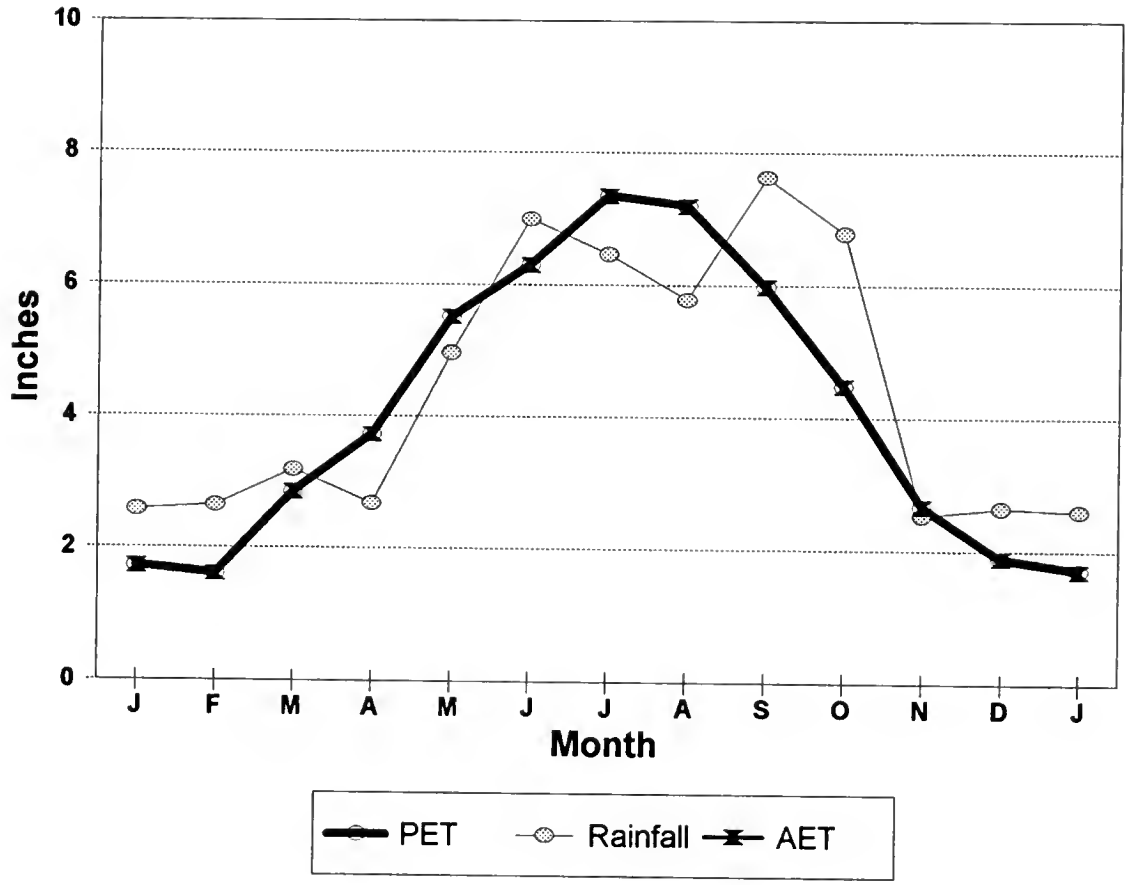


Figure 5-18. Thirty year water balance for Stuart 1 N (06-8620) using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

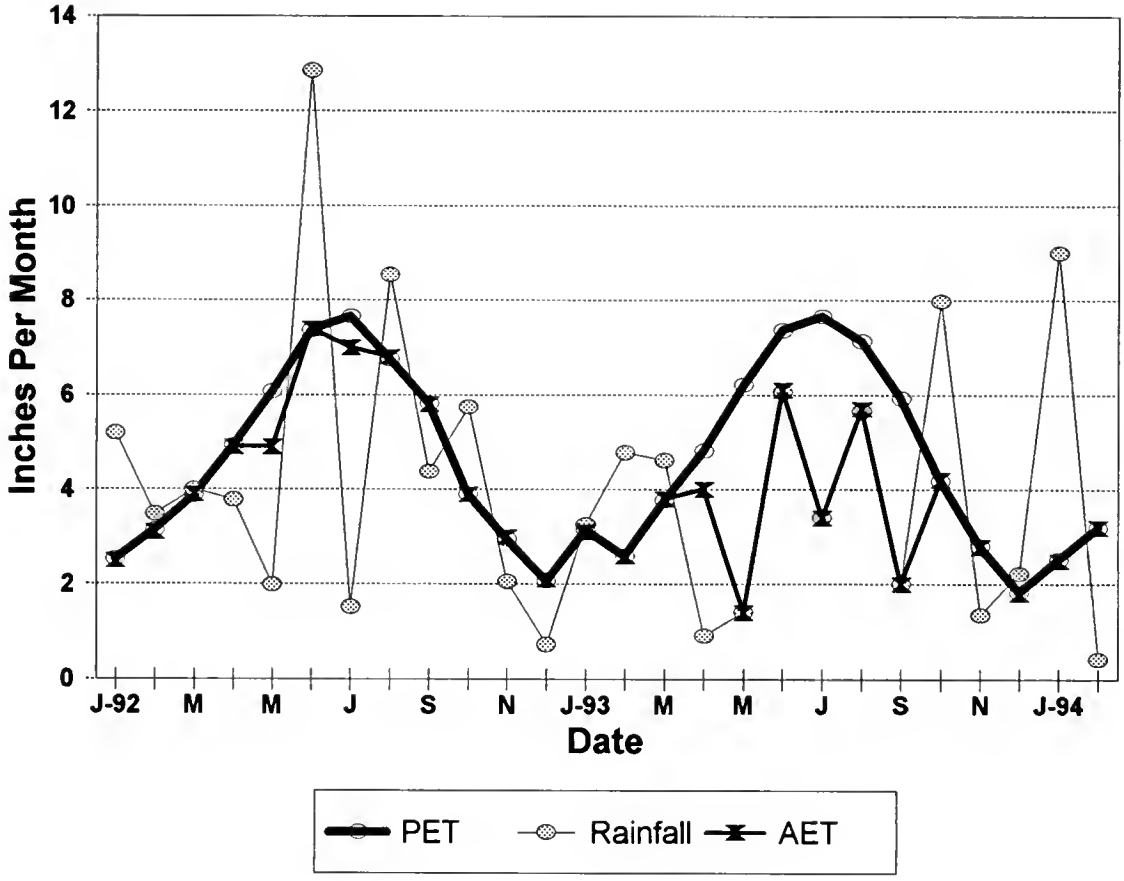


Figure 5-19. Water balance for Gainesville 2 WSW (02-3321) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

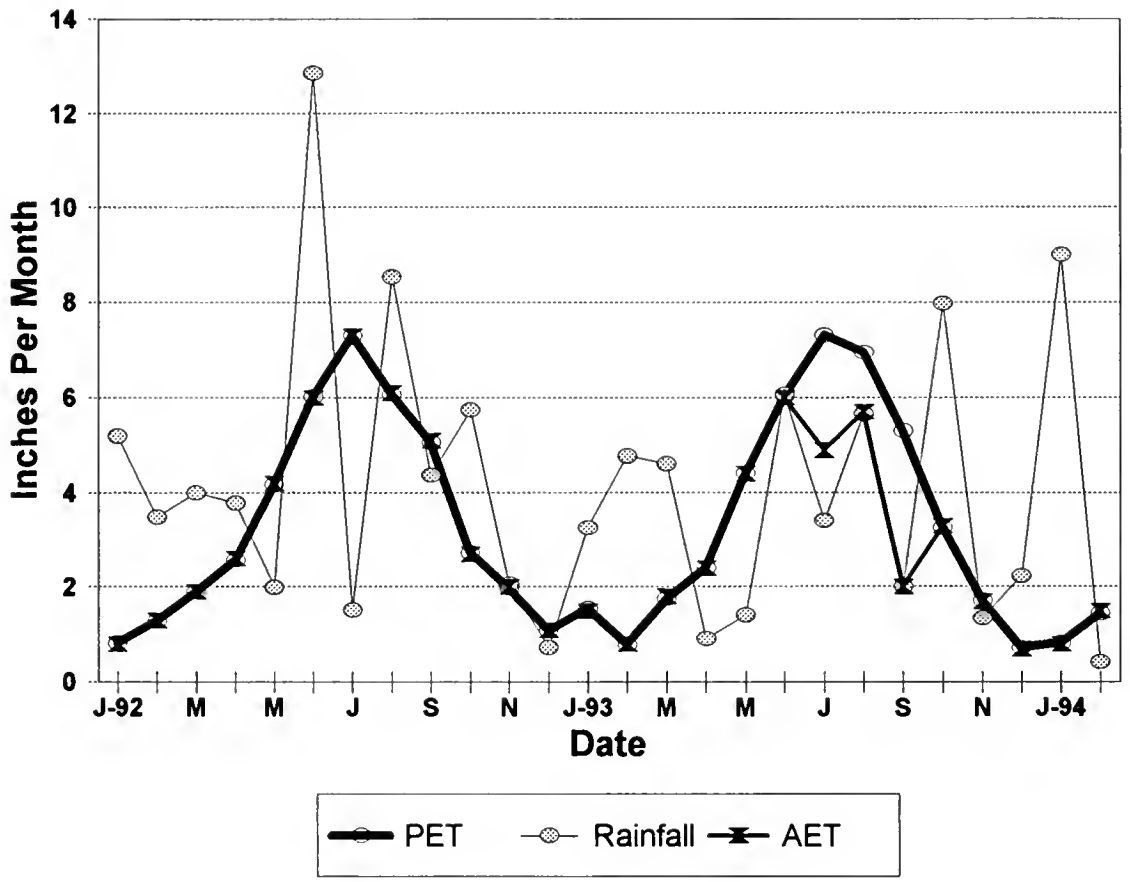


Figure 5-20. Water balance for Gainesville 2 WSW (02-3321) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

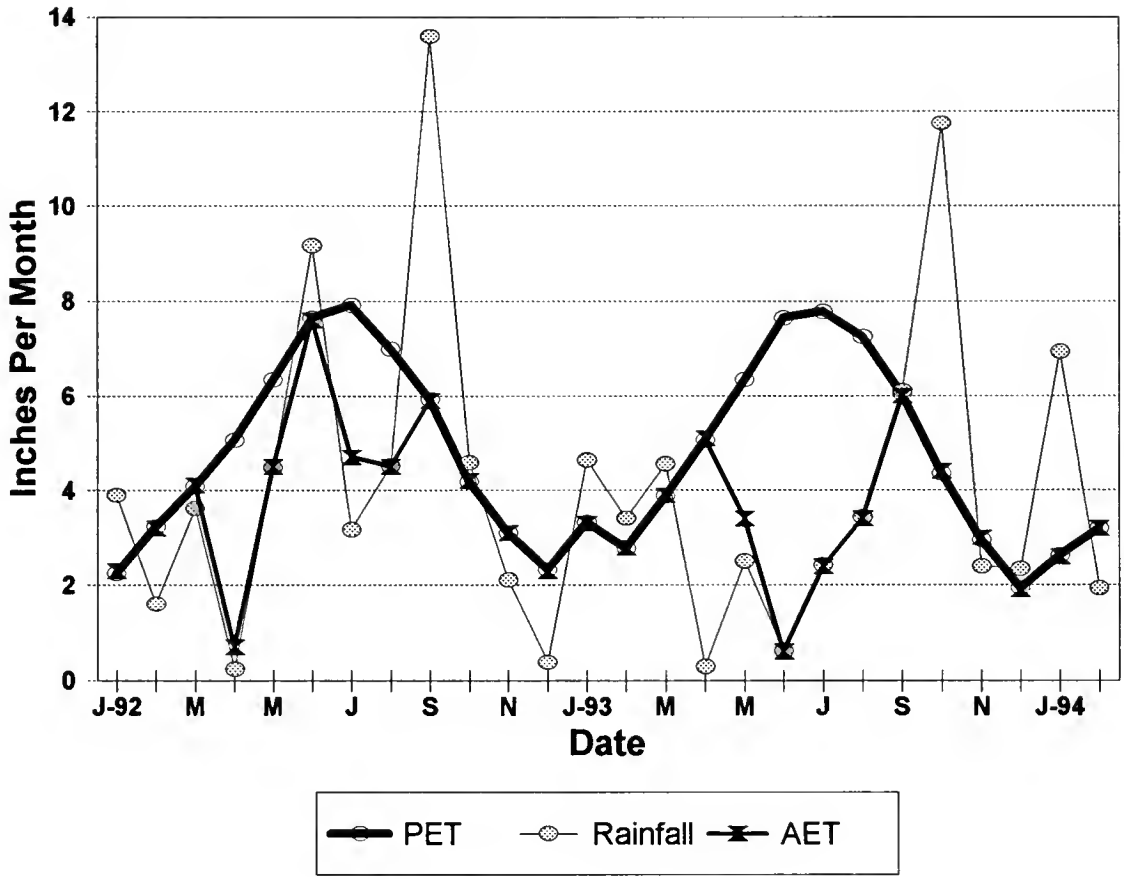


Figure 5-21. Water balance for St. Augustine WFOY (02-7826) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

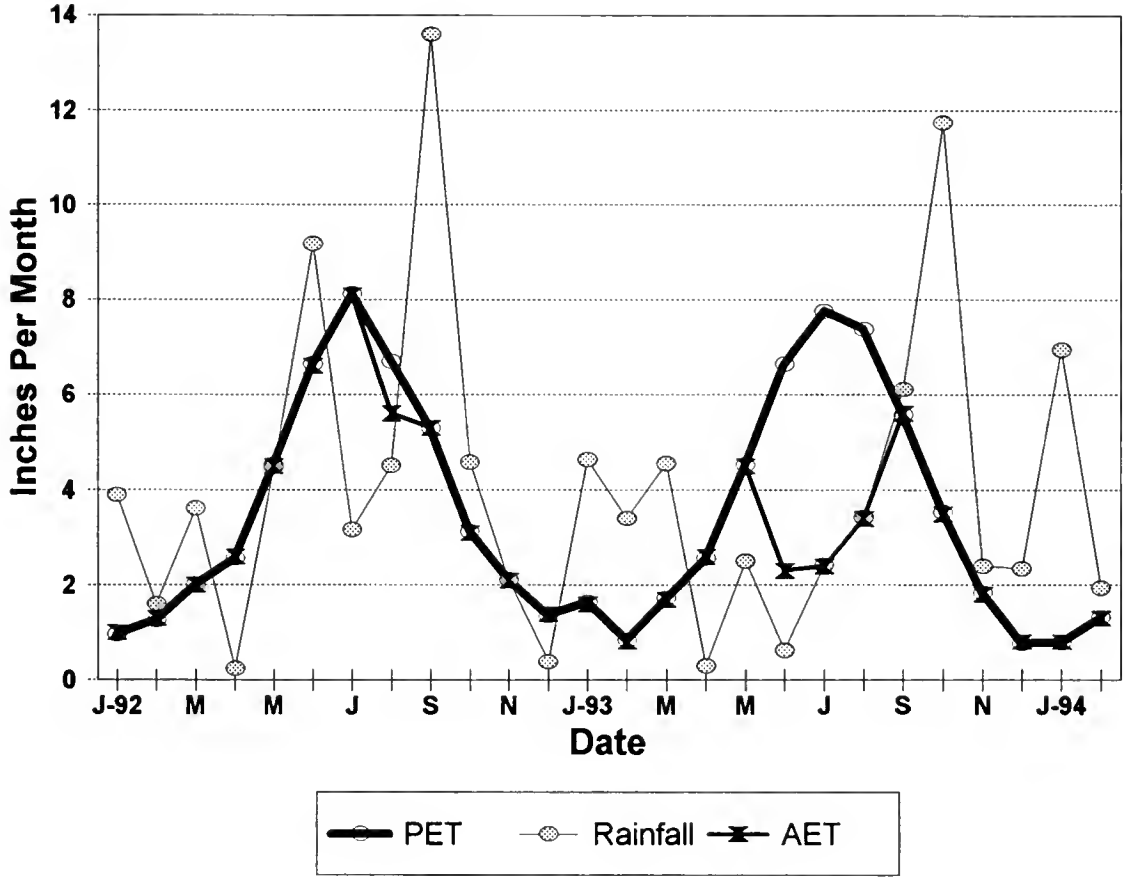


Figure 5-22. Water balance for St. Augustine WFOY (02-7826) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

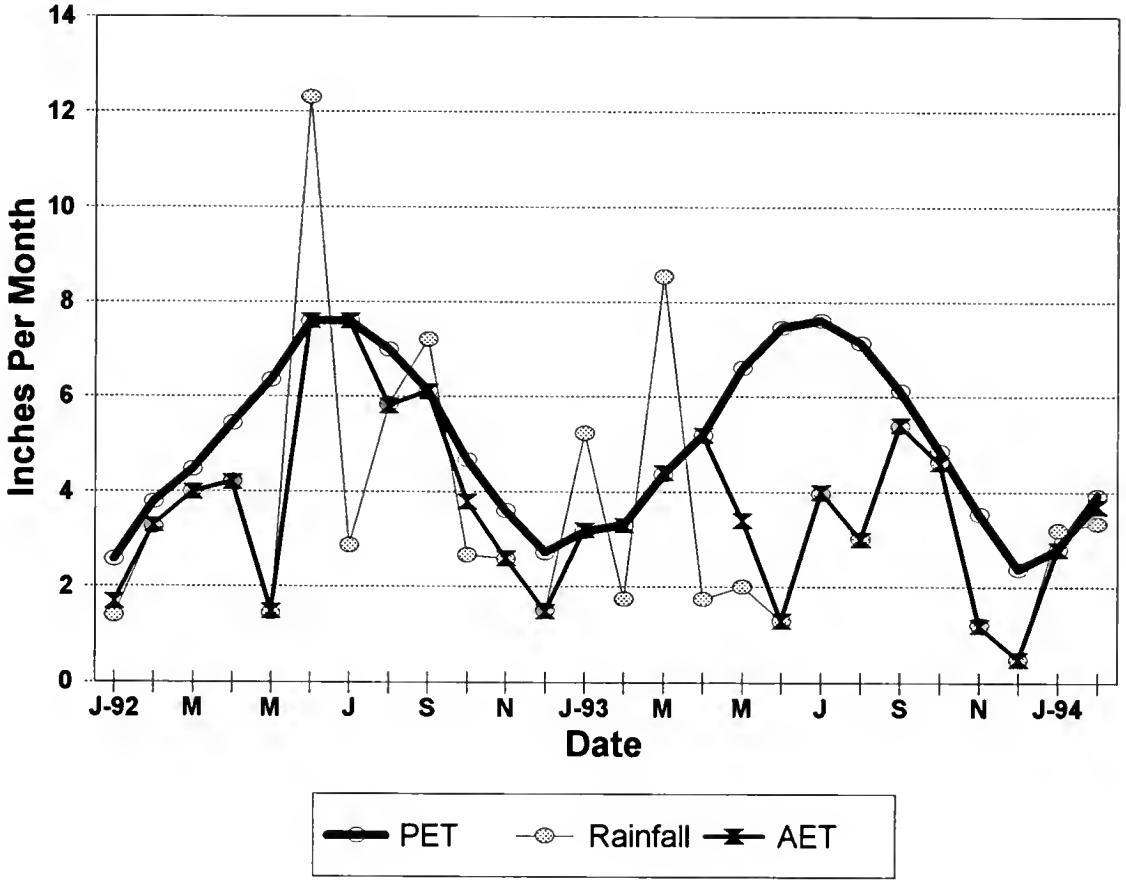


Figure 5-23. Water balance for Melbourne WSO (04-5612) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

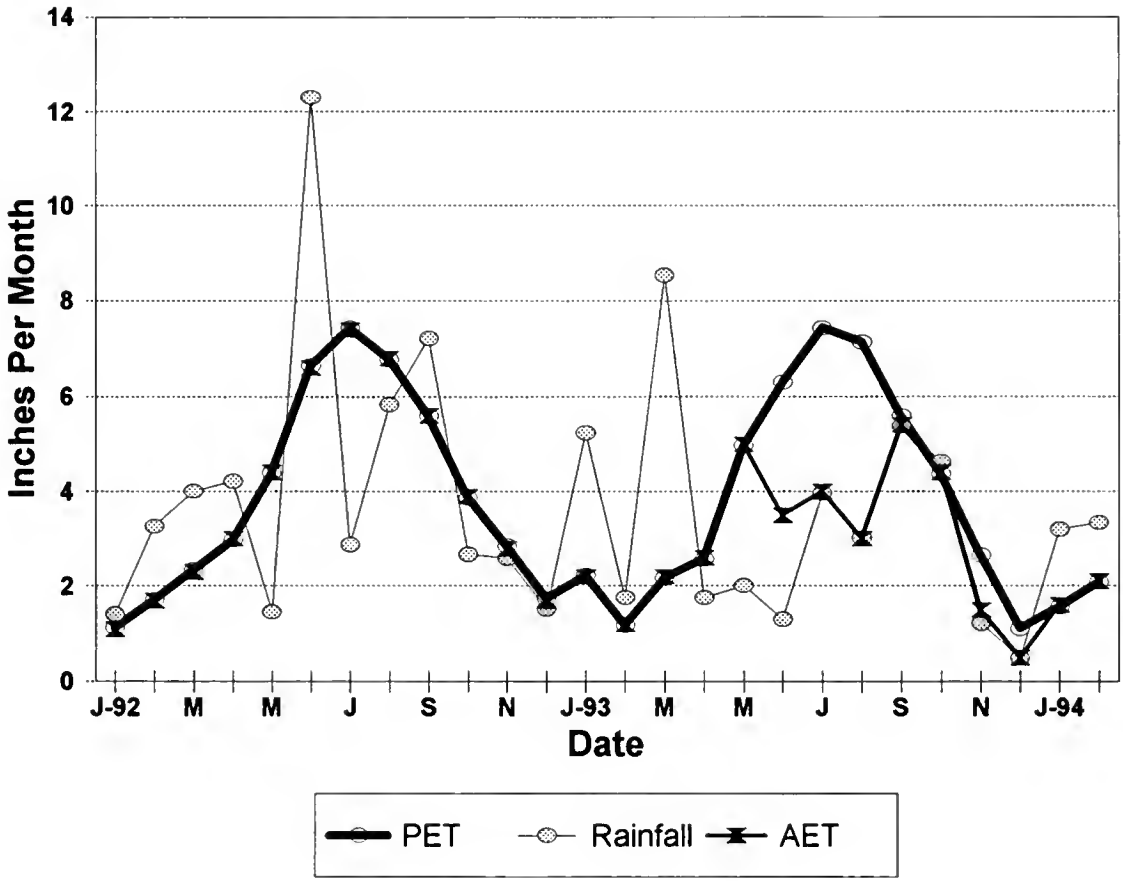


Figure 5-24. Water balance for Melbourne WSO (04-5612) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

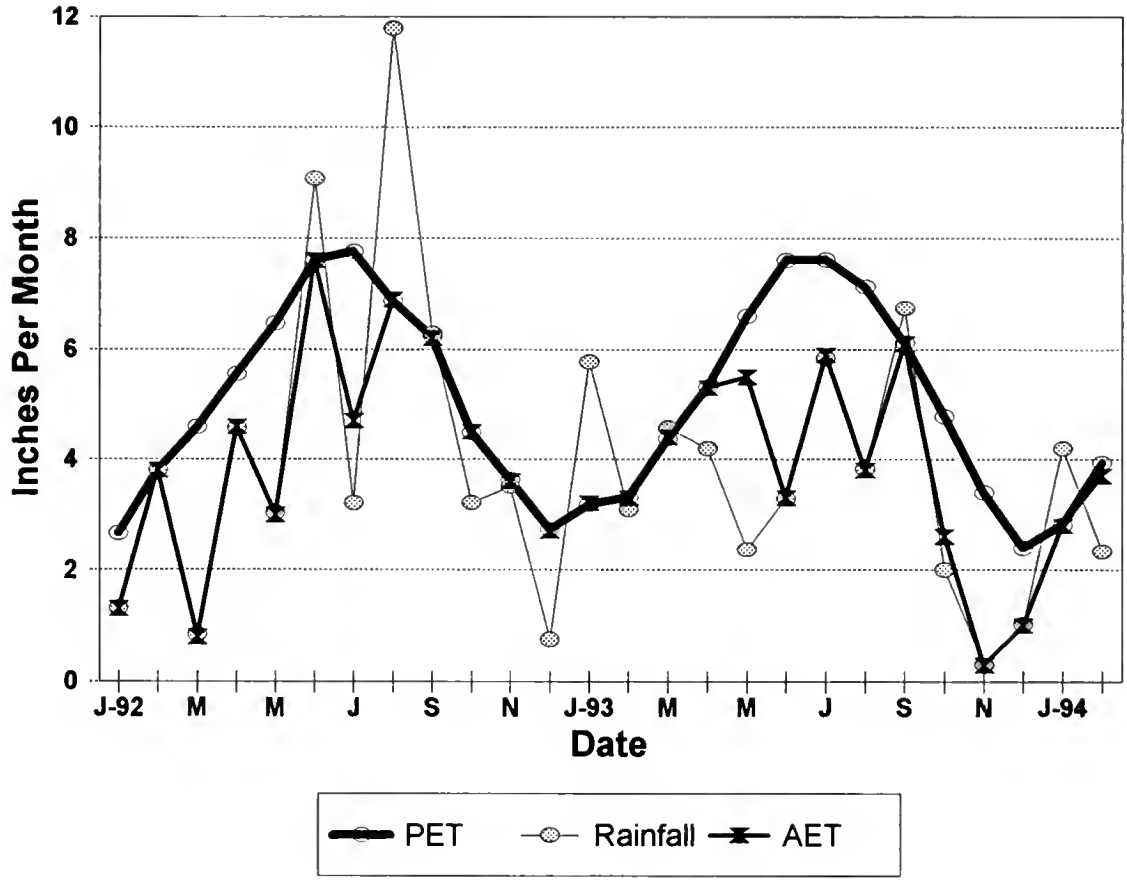


Figure 5-25. Water balance for Mountain Lake (04-5973) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

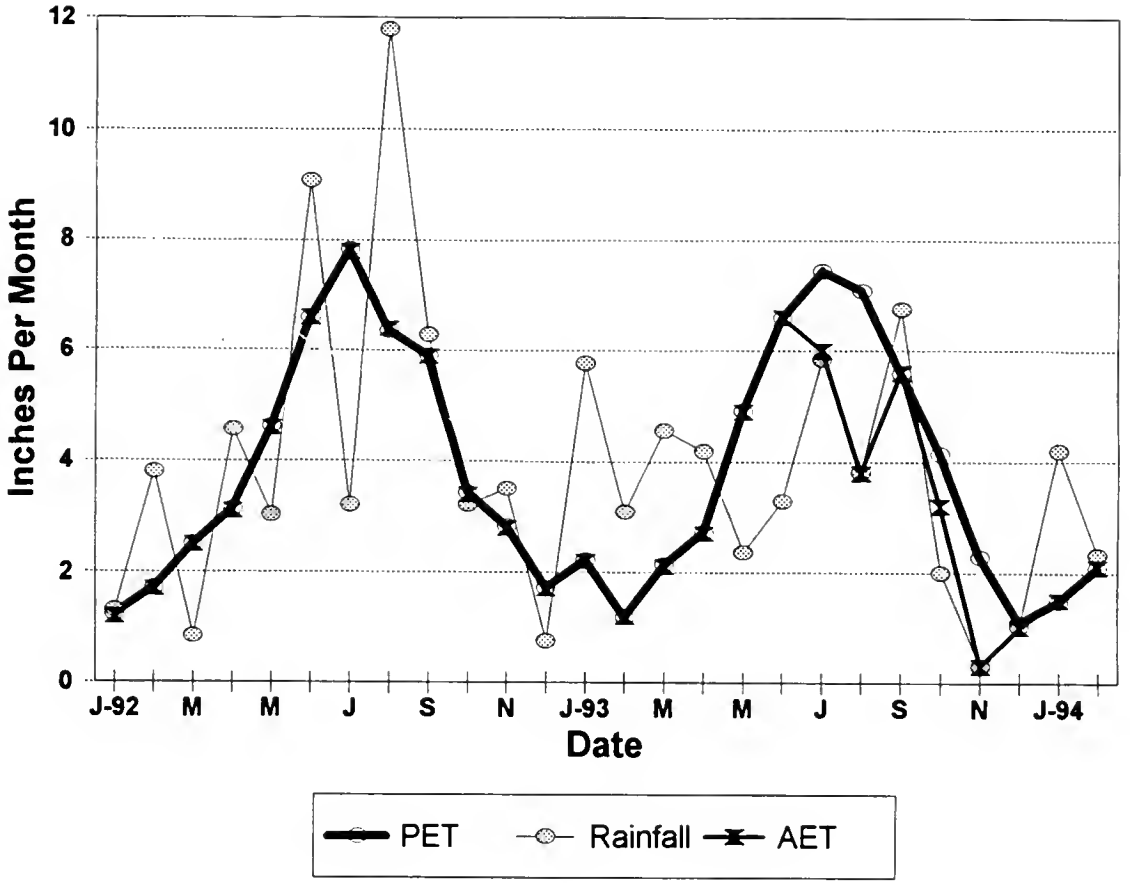


Figure 5-26. Water balance for Mountain Lake (04-5973) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

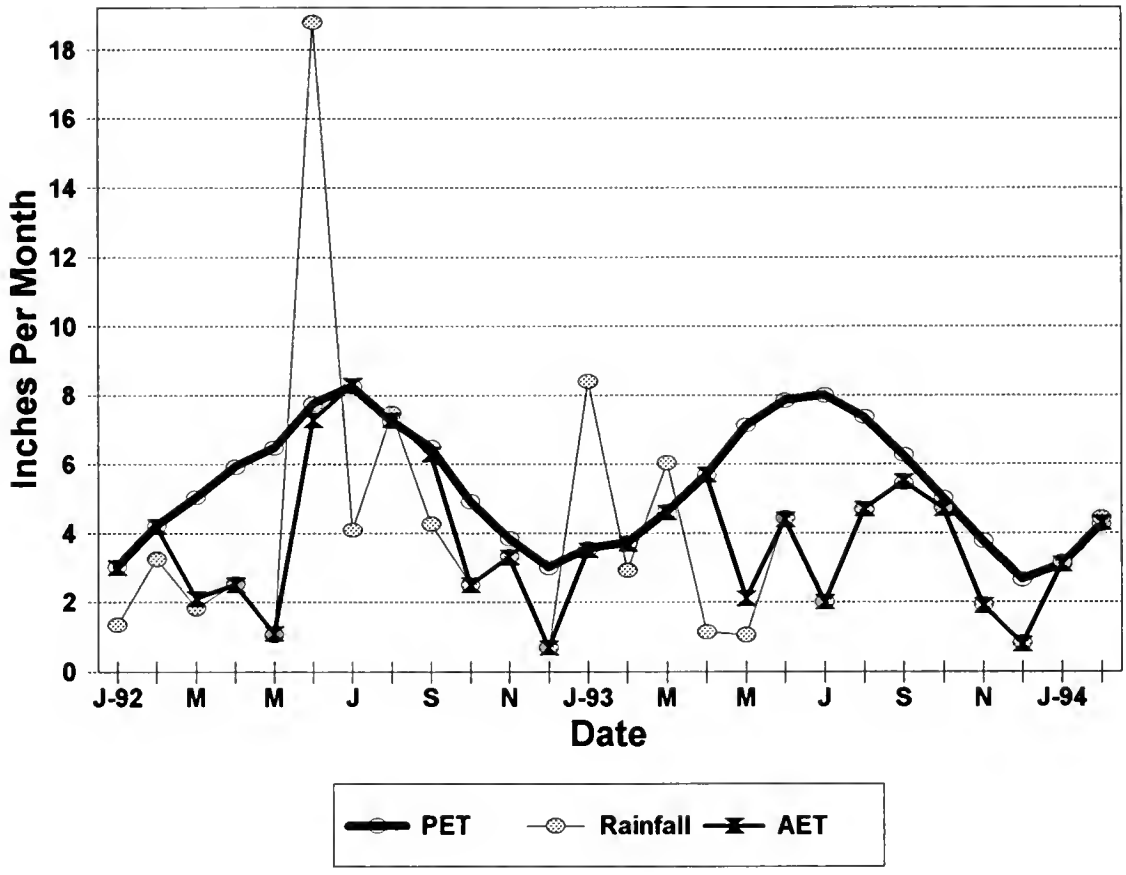


Figure 5-27. Water balance for Okeechobee HRCN Gate 6 (04-6485) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

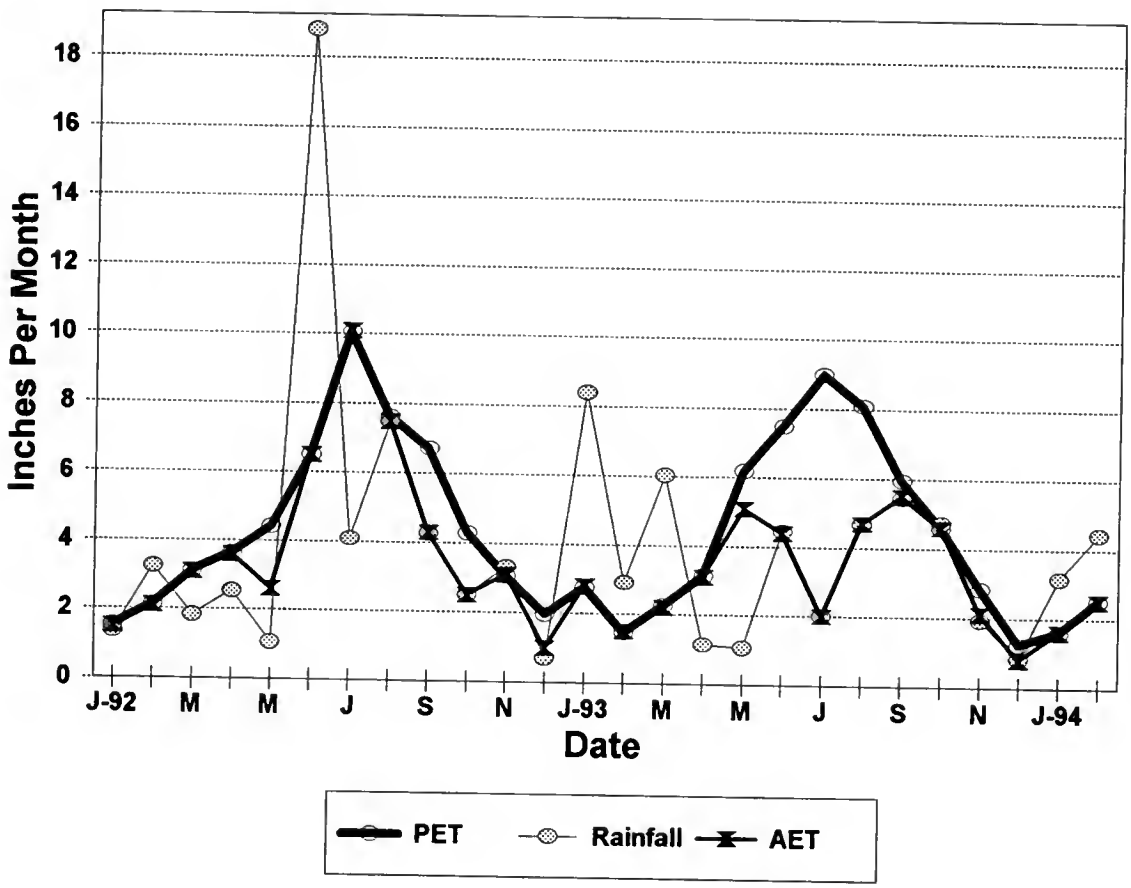


Figure 5-28. Water balance for Okeechobee HRCN Gate 6 (04-6485) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

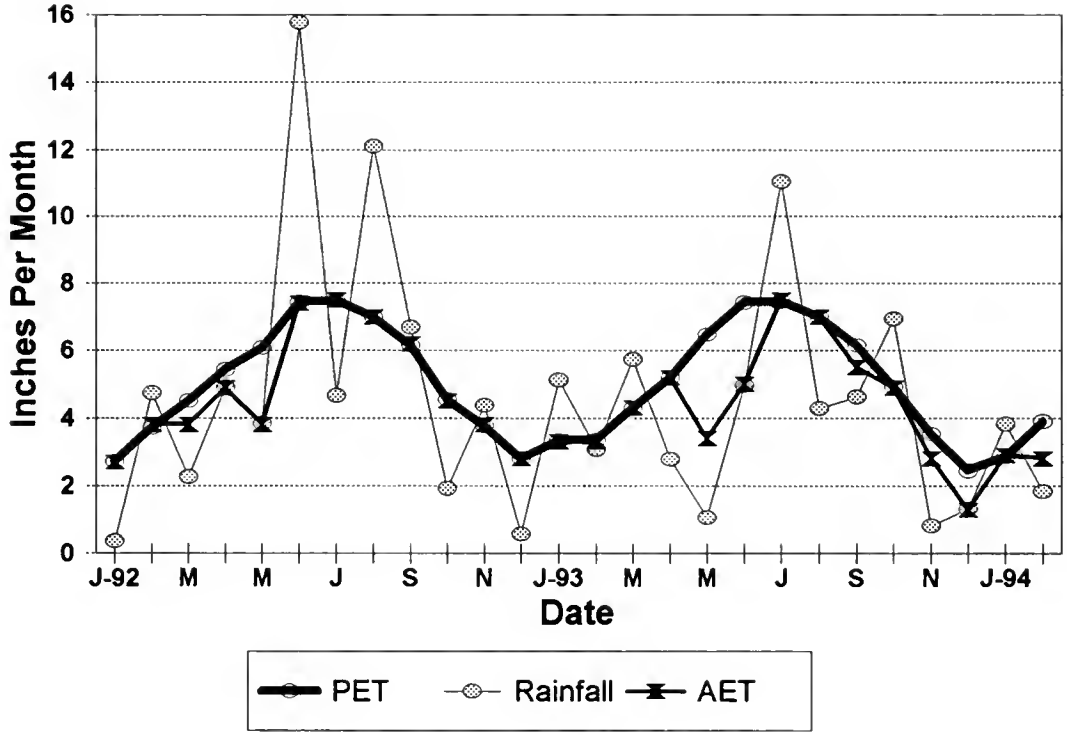


Figure 5-29. Water balance for Archbold Biological Station (04-0236) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Stephens-Stewart method.

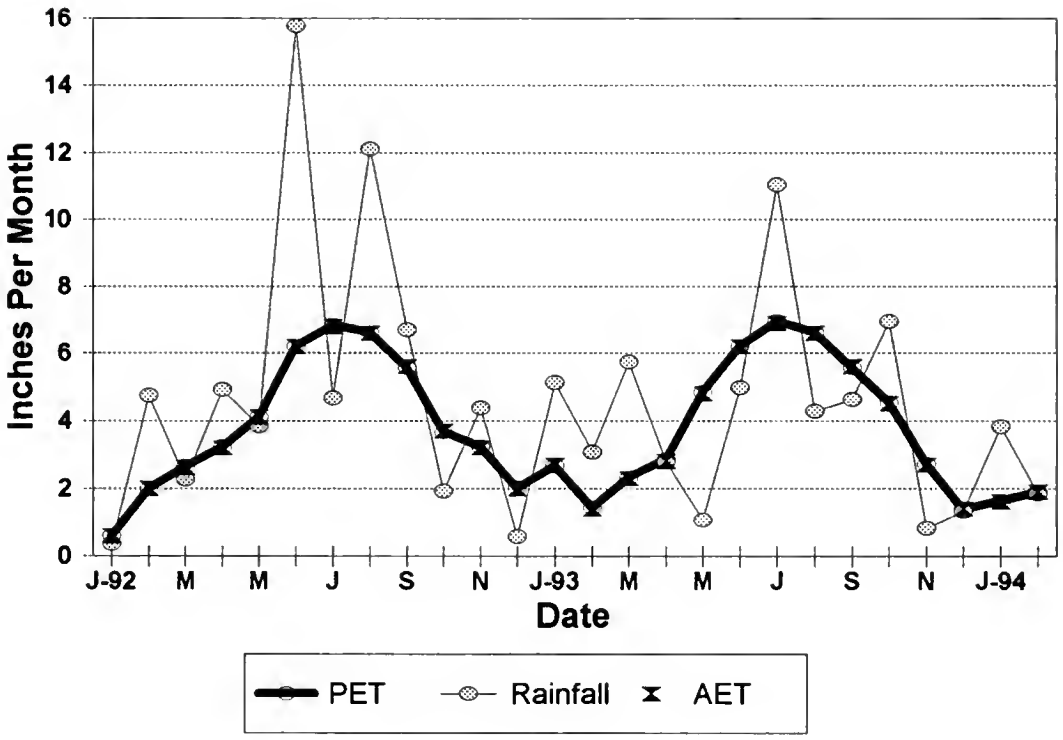


Figure 5-30. Water balance for Archbold Biological Station (04-0236) during the period, January 1992 to February 1994, using a 6 inch soil moisture storage model. PET values were calculated using the Thornthwaite method.

Figure 5-31. Average numbers of snails found by timed and quadrant collection methods in southern (a), central (b) and northern (a) Florida. Snail counts for each region were normalized by dividing the total number of snails collected on a given date by the number of sites for which collections were made.

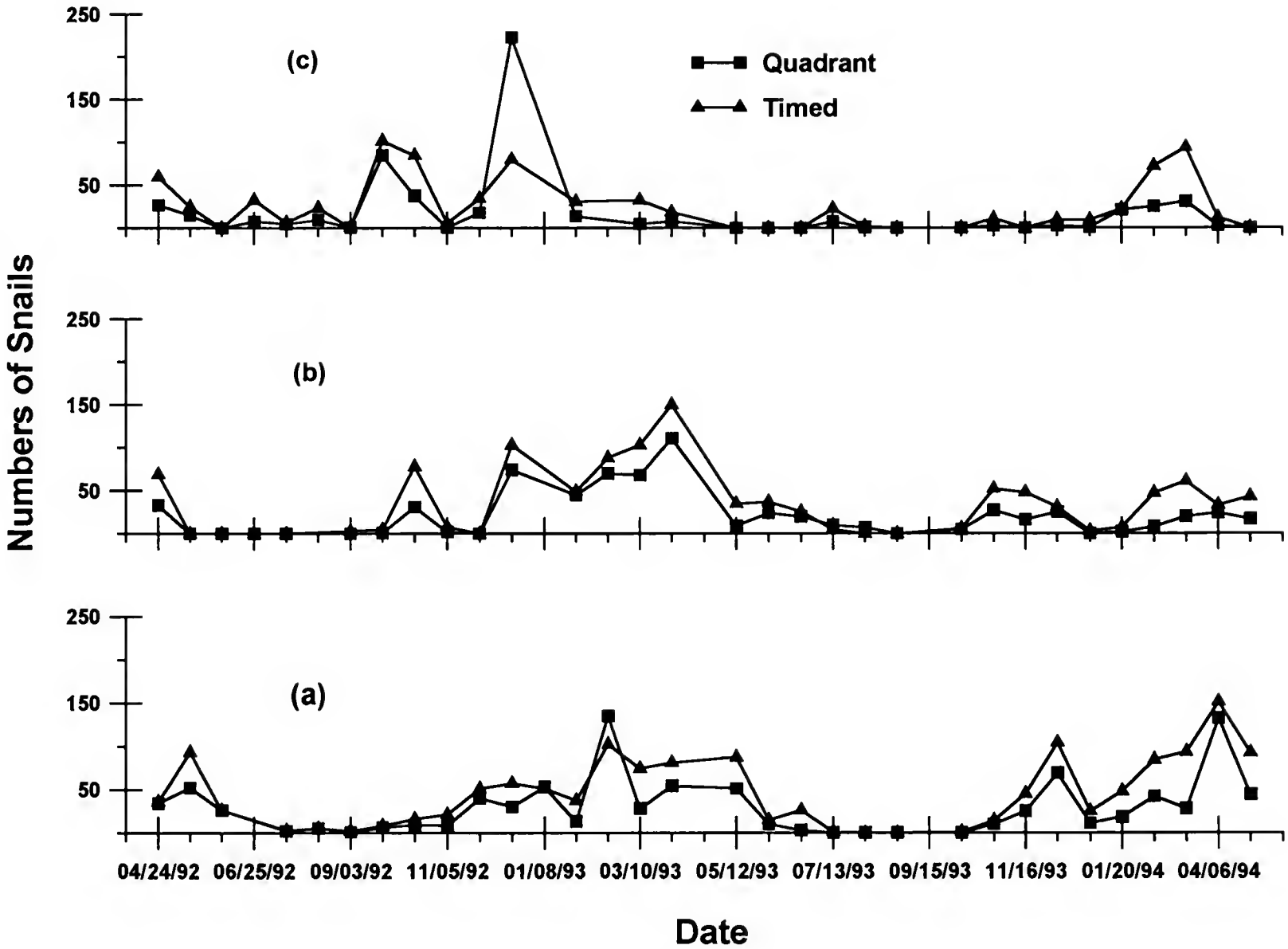
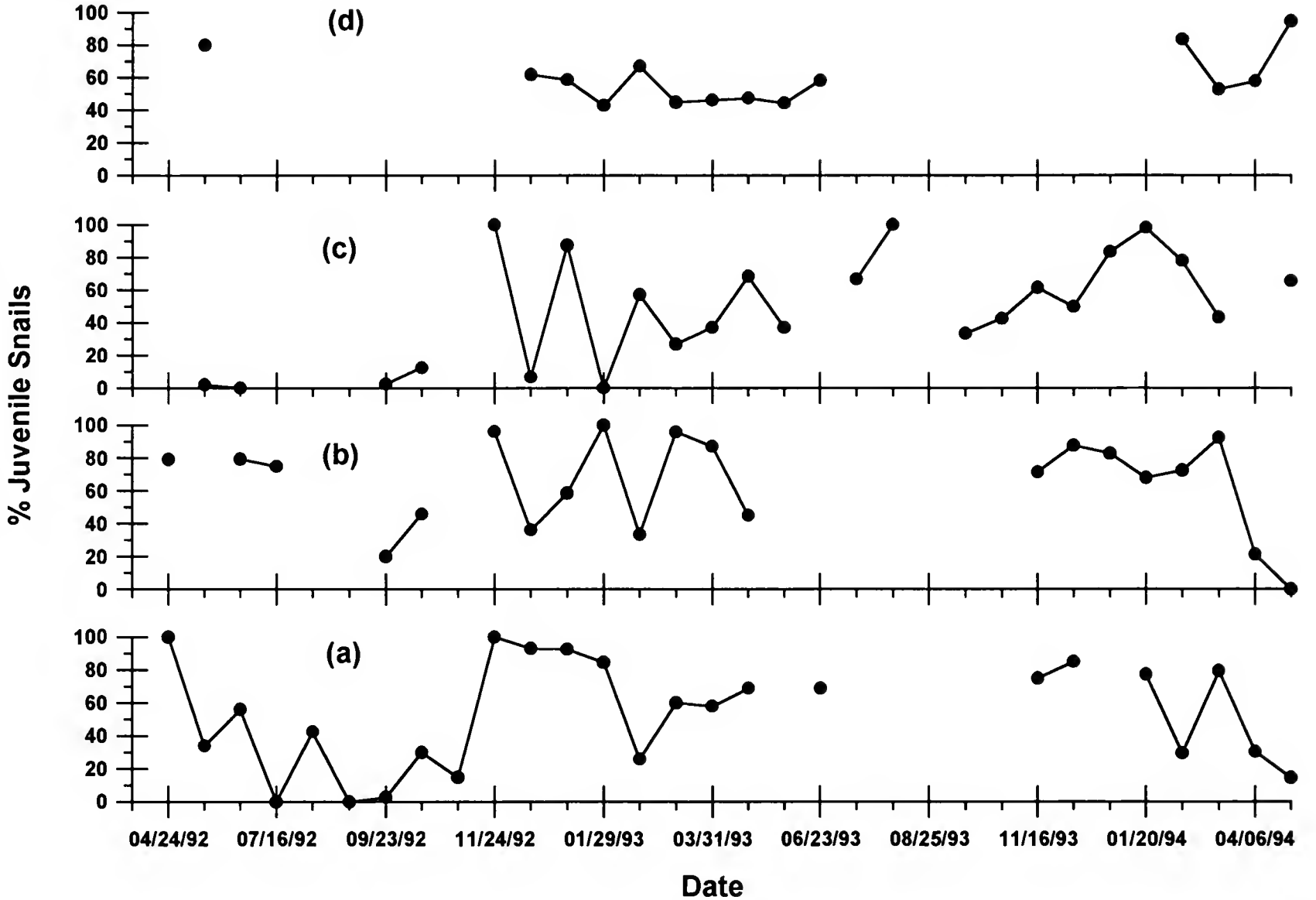

(c)
(b)
(a)
Quadrant
Timed
250
150
50
Numbers of Snails
04/24/92
06/25/92
09/03/92
11/05/92
01/08/93
03/10/93
05/12/93
07/13/93
09/15/93
11/16/93
01/20/94
04/06/94
Date

Figure 5-32. Percentage of snails in each collection that were juvenile (≤ 4 mm). Brighton Seminole Indian Reservation, site 1 (a) and site 2 (b); Rio Ranch, site 1 (c) and site 2 (d).


(d)
(c)
(b)
(a)
100
80
60
40
20
0
% Juvenile Snails
04/24/92
07/16/92
09/23/92
11/24/92
01/29/93
03/31/93
06/23/93
08/25/93
11/16/93
01/20/94
04/06/94
Date

CHAPTER 6
SUMMARY AND CONCLUSIONS

Fascioliasis, caused by infection with the liver fluke, *Fasciola hepatica*, remains one of the most important diseases of grazing ruminants throughout much of the world. This is also true in Florida, where over 90% of the state's 1.1 million beef brood cows are pastured in areas enzootic with *F. hepatica*. Approximately 68% of these cows are infected with liver flukes and economic losses in Florida are estimated at greater than 11 million dollars annually.

Prior to this study, no data was available on the seasonal transmission dynamics of *F. hepatica* in southern Florida where more than 66% of Florida's beef cattle are pastured. This was important because of conjectural evidence suggesting that transmission dynamics in the southern portion of the state may differ from that in the northern portion. If differences in the seasonality of transmission do exist, then current control recommendations based upon data from northern Florida would be inappropriate for cattle in southern Florida. To resolve this issue, the epidemiology of *F. hepatica* was studied over 2 years in northern, central, and southern Florida. The seasonal transmission dynamics of fascioliasis is often established for a particular area through the monthly

slaughter of sentinel animals ("tracers") grazing known fluke-infested pastures, but this is an expensive process. Using tracer animals in this 2-year study would have cost greater than $500,000. This level of funding would be difficult if not impossible to procure. Therefore, an alternative approach was required.

Patterns of *F. hepatica* transmission are the direct result of interactions between the snail intermediate host, the parasite and the environment. Therefore, data on the bionomics and infection prevalences of these snails can be used to predict changes in seasonal pasture contamination levels and the ensuing risk to grazing livestock. Thus, it is possible to determine seasonal transmission dynamics without using expensive tracer animals, particularly if a method for rapidly testing large numbers of snails for infection is available. This alternative approach was taken in this project because it has the advantage of allowing the investigation of more sites at a far lower cost than can be done using sentinel animals.

In this project, the bionomics of *Fossaria cubensis* populations were studied at 3 week intervals on 7 cattle ranches over a 2-year period. Snails were collected and brought back to the laboratory where they were counted, measured, and during year-2 of the project, assayed for infection with *F. hepatica*. Techniques used in previous studies to detect *F. hepatica* infections in snails lacked the

sensitivity, specificity and efficiency necessary to obtain accurate and meaningful data. To overcome this problem, a DNA probe assay was developed. This DNA probe (pFh5), contains 2 124 bp repeat sequences that belong to a large family of closely related (approximately 90% identity) 124 bp repeats. Quantitative dot blot analysis demonstrated that this family of 124 bp repeats constitute approximately 15% of the *F. hepatica* genome. Using this DNA probe, infected snails can be detected immediately following miracidial penetration, thus a sensitivity equivalent to the minimum biologic unit of the parasite is achieved. The probe is also highly specific; it does not cross hybridize with DNA of *Fascioloides magna*, *Paramphistomum liorchis*, or *Heterobilharzia americana*, trematodes that share the same snail intermediate hosts and have overlapping enzootic ranges with *F. hepatica*.

However, before a nucleic acid probe can be used in a large epidemiologic survey, sample processing must be made both time and cost efficient. Therefore, a quick, inexpensive and safe assay using chemiluminescent detection also was developed. The entire assay was performed in a clinical parasitology laboratory without the need for specialized equipment or facilities that only would be available in a recombinant DNA laboratory. The assay is also very quick and inexpensive to perform. The entire assay can be completed for the low cost of about $0.33 per snail and takes only about 3 min per snail to complete when large numbers of snails (~400)

are assayed at a time. Under these assay conditions, sensitivity of the probe assay is 100% and specificity is greater than 99%.

Using this DNA probe assay, more than 5,000 snails from 6 cattle ranches were assayed for infection during the second year of this study. Infection prevalence for individual ranches varied from 0.1% to 3.1%. Additionally, the prevalence of infection increased for each successive size class reaching 18.5% for 9 and 10 mm snails.

Results of this study demonstrated that the epidemiology of *F. hepatica* can be determined without the use of tracer calves. Seasonal transmission dynamics of liver flukes are virtually the same for all of Florida, with transmission occurring predominantly in the winter and spring. Wet summers followed by cool, wet weather in the early autumn could result in significant levels of transmission occurring by December. Summer transmission will rarely occur in Florida. Although snail habitats remain wet throughout the summers of most years, ecological conditions of *Fossaria* habitats are not conducive to snail activity.

Fluke control recommendations for beef cattle in Florida are predicated on the occurrence of this summer gap in fluke transmission. Flukicidal treatment of cattle in the early autumn will kill virtually all of the adult flukes thus preventing the shedding of large numbers of fluke eggs onto pasture when snails emerge from summer aestivation with the

onset of cooler weather. Because newly emerged snails and their subsequent offspring are not likely to become infected if cattle have been treated, fluke transmission to cattle is greatly reduced during the following winter and spring. However, if eggs can survive on pasture for any length of time, flukicide would need to be given far enough in advance of the return of snails to gain the full benefit of this treatment. Earlier recommendations regarding the timing of autumn treatment did not take the possibility of egg refugia on pasture into account. Therefore, as part of this project to determine the epidemiology of *F. hepatica* in Florida a set of experiments were done to determine the ability of eggs to survive, develop and hatch on pasture during the summer months.

Results of those experiments demonstrated that eggs of *F. hepatica* can survive and develop during the summer even after 28 days on pasture. Accordingly, treatment should be given at least 4 weeks prior to the emergence of snails from aestivation to allow sufficient time for eggs already on pasture to hatch and miracidia to die. Treatment for flukes in Florida should therefore be given in the late summer, mid-August to early September, to obtain maximum benefit. This is 1 to 2 months sooner than recommended previously.

Results from this study do not help resolve the issue of whether or not to treat cattle a second time in the spring. Previous recommendations were to treat cattle in the spring

(in addition to but never instead of autumn treatment) in unusually wet years or on properties with a history of severe fascioliasis. Without data on the cost-benefit of spring treatment, these recommendations remain unchanged. The problem with spring treatment is that flukicidal drugs do not kill juvenile flukes. Therefore, a large percentage of the flukes that are infecting cattle in the spring will not be killed, even with a full dose of clorsulon (drug of choice for spring treatment). Even so, if cows harbor large fluke burdens, they and their calves will most likely benefit from treatment.

Future research into the epidemiology of bovine fascioliasis in Florida should address several important issues that remain unresolved. Better data is needed on the microclimate of snail habitats and the relationship between this data and site-specific and regional-specific meteorological data. It should be possible with several years of precise data to develop a simple model to predict relative fluke risk for individual years. A prerequisite to developing and validating this model will be several years of research that combines all parameters used to measure liver fluke transmission. Thus snail bionomics, snail infection prevalence, tracer calf fluke acquisition, microclimate conditions and meteorological data must all be included.

If such a model is developed, it would probably be of great benefit to the study of *F. hepatica* epidemiology

throughout the world. By correlating fluke acquisition with the other parameters studied, future studies done elsewhere could eliminate the use of tracer animals. This would dramatically reduce the cost of both determining local transmission dynamics and developing models that are regionally appropriate.

Another area of future research is in the optimization of the DNA probe assay, so that the maximum amount of information can be obtained from the data. Using radioisotopic detection, signal intensity increases in a logarithmic fashion over time. Therefore, signal intensity can be measured and used to estimate the maturity of the infection giving further information on the bionomics of the infected snail populations on pasture. This was not done in this study because a chemiluminescent detection system was used. One way to gain advantage of both systems is to first perform the assay using chemiluminescence when screening snails collected from the field for infection. Because only half the sample is used in this assay, all positive samples then can be assayed a second time using radioisotopic detection (or other new detection systems that allow for quantitation of signal such as fluorescence). Using standard curves that would be established by assaying large numbers of experimentally infected snails, the approximate maturity of infections could be determined for individual snails. Additionally, by quantitatively measuring all weak positives, many of these

could be correctly classified as true weak positives or false positives.

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BIOGRAPHICAL SKETCH

Ray M. Kaplan was born October 27, 1960 in Brooklyn , New York. He is the youngest son of Aaron and Elaine Kaplan. Ray grew up in Monsey, New York, a suburb of New York City. He graduated from Spring Valley High School in 1978.

Ray received his Bachelor of Science in Dairy Science from Virginia Polytechnic Institute and State University in 1983. Ray participated in the cooperative education program, working at the United States Department of Agriculture (USDA) in the Ruminant Parasitic Disease Lab and the Milk Secretion and Mastitis Lab.

Ray continued his work at the USDA prior to attending the Virginia-Maryland Regional College of Veterinary Medicine. He graduated with his Doctor of Veterinary Medicine in 1988. He then practiced veterinary medicine in a mixed species practice in central Pennsylvania. He was accepted into the University of Florida, College of Veterinary Medicine for graduate studies in 1990.

Dr. Kaplan married Holly S. Mueller in June of 1986. They have two children, Elyssa Rose born March 20, 1990, and Harrison Michael born January 4, 1995.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles H. Courtney, Chair
Professor of Veterinary Medicine

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